

DOES INDUCED RESISTANCE OF BACTERIA TO
ONE ANTIBIOTIC RESULT IN SIMULTANEOUS
SENSITIVITY CHANGES TO OTHER
ANTIBIOTICS?

BY

W. J. KAIPAINEN

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FROM THE DEPARTMENT OF SEROLOGY AND BACTERIOLOGY,
UNIVERSITY OF HELSINKI

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ACADEMIC DISSERTATION

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Kirjapaino Oy. Liike

To my wife

Preface

I wish to express my profound gratitude to my respected teacher, the Chief of the Department of Serology and Bacteriology of Helsinki University, Prof. K. O. Renkonen, M.D. I have had the opportunity of carrying out my work in the department led by him, one in which the atmosphere is known to be very propitious to scientific work. Prof. Renkonen has shown great interest in my work and has always been prepared, readily and regardless of time or trouble, to give me valuable advice at all stages of my work.

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Helsinki, December 1950.

The Author.

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I Introduction

After the finding (Chain, Florey *et al.* 6) of the excellent therapeutical potentialities of the penicillin discovered by Fleming (20) in 1929 the study of antibiotics increased rapidly. The studies resulted in streptomycin described by Waksman *et al.* (48, 54) in 1944, followed by chloromycetin described by Burkholder, Ehrlich *et al.* (13) in 1947, aureomycin described by Duggar (12) in 1948, and finally in terramycin described by Finlay *et al.* (19) in 1950. Although a considerable number of pathogenic bacteria can be attacked with these substances, one of their characteristic drawbacks is that the bacteria may grow resistant to them. The future will show to what extent the antibiotics used today will lose their importance because of the increased resistance of bacteria. The endeavour is to counter by every possible means this increase in the resistance of bacteria. Sensitivity determinations and a sufficient dosage of the correctly chosen antibiotic are naturally fundamental conditions for preventing an increase in the resistance of the bacteria.

The prevailing conception is that the increased resistance of bacteria to any one antibiotic is not accompanied by a simultaneous strengthening of resistance or sensitivity to the other antibiotics. However, the author found an indication of such simultaneously increased resistance when treating a patient with aureomycin for an infection of the urinary tract caused by *E. coli*. The resistance of the *E. coli* strain isolated at a later stage of the treatment was increased both to aureomycin and chloromycetin, as compared with the original *E. coli* strain. Dihydrostreptomycin sensitivity was the same with both strains. Provided the original and the later strain were the same, it was possible that chloromycetin resistance and aureomycin resistance had increased simultaneously as the result

of aureomycin therapy. Later on, in fact, the author noticed in experiments in vitro that the increase in resistance to one antibiotic was accompanied by a simultaneous increase in resistance to other antibiotics also. As a result of these experiments the correctness of the prevailing conception seemed open to doubt.

II Review of the Question of Resistance

The increased resistance of bacteria to antibiotics has been the subject of lively investigation. In part, the investigations have been concerned with the way in which the bacteria grow resistant to antibiotics, in part with the observations concerning the persistence of the resistance and other properties of the resistant strains. Abraham *et al.* (1) were the first to describe the increased resistance of staphylococci to penicillin. They grew staphylococci in broth containing penicillin; after 9 weeks they found that the resistance had increased 30-fold, and after the cultivation had continued for another seven weeks the increase was 1000-fold.

Origin of increased resistance

Simultaneously with the discovery that the resistance to antibacterial substances increased, investigations were carried out into the theory of the origin of the increase. Two different hypotheses have been advanced:

1. The increased resistance of the bacteria is the final result of adaptation to a certain growth-inhibiting microbial agents.

2. The increase in resistance takes place by way of genetic mutation, the effective agent constituting a selective factor only.

The theory that the increase in resistance takes place through spontaneous mutation was first advanced by Luria and Delbrück (38), who studied the increased resistance of *E. coli* to bacteriophages. Demerec (8) explained the increased resistance of *Staphylococcus aureus* to penicillin as taking place through spontaneous mutation in which penicillin only selectively eliminates sensitive individuals. Spontaneous mutation is also supported by Witkin (57), who studied the effect of ultraviolet rays on *E. coli*.

Oakberg and Luria (41) studied the increased resistance of *Staphylococcus aureus* to sulfonamide and were of the opinion that here too a spontaneous mutation was in question.

In several investigations into the increase in the resistance of bacteria to the various antibiotics Demerec (4, 8, 9, 10) has continuously emphasised that the growing resistance is a result of spontaneous mutation and that antibiotics always only play the selective role. Vesterdal (53), studying the increased resistance of staphylococci to penicillin, came to the conclusion that penicillin provokes the substances contained in agar to produce a mutation resulting in increased resistance. Bellamy and Klimck (3) studied the increase in the resistance of *Staph. aureus* to penicillin and were of the opinion that a mutation induced by penicillin was in question.

Few researchers have opposed the theory of spontaneous selection. Among them may be mentioned Eriksen (17) who has tried to show that Demerec's theory is not correct. Although Eriksen's investigations seem plausible there are phenomena that to some extent favour the theory of spontaneous mutation. Such, for instance, are the considerable increases in resistance without the presence of antibiotics. Foster and Woodruff's (21) daily sub-cultures of a *Staphylococcus aureus* strain in broth yielded variants of which the resistance to penicillin exceeded the original. Spink and Ferris (51) report similar results in their tests with *Staphylococcus aureus* strains. In these tests they grew, for some time, a penicillin-resistant *Staphylococcus aureus* strain, first in Gladstone's medium and subsequently in broth. The original sensitivity to penicillin was 2 units/ml and after the sub-cultures 60 units/ml. The resistance to penicillin, therefore, had increased without the presence of penicillin. A further experiment was carried out with another *Staphylococcus aureus* strain resistant to penicillin. Their method was to divide the strain into two parts, of which one was grown in broth, the other in broth containing penicillin. The original sensitivity was 20 units/ml. In penicillin-free cultures the resistance increased to 400 units/ml, whereas in the broth containing penicillin it amounted to 600 units/ml. It would have been of interest to see how the resistance of these strains would have changed had they been cultivated without penicillin simultaneously in media of other types.

Patterns of resistance to the various antibiotics.

In several of his works on increased resistance, in which Demerec (4, 8, 9, 10) emphasises that the increase in resistance takes place through spontaneous mutation and considers the role of the antibiotic as selective, he has assumed the existence of two types of patterns in the increase in resistance, both of which are dependent, however, on the antibiotic in the first place. The first is the penicillin type, in which complete resistance is achieved through several genetic steps. The first step (due to the selective action of the antibiotic) yields material for the second step, and so on until complete resistance is achieved. In this pattern type complete resistance is never achieved by a single mutation. This type comprises penicillin, aureomycin, chloromycetin, terramycin and neomycin. The second, or the streptomycin pattern, may become resistant by a single step or through several steps. Forms that grow resistant by a single step are likely to be problematic to clinicians from the streptomycin treatment point of view.

On the duration of increased resistance.

Increased resistance has been found both to persist and to disappear when the strain that has become resistant is cultivated without the antibiotic. If stored at a low temperature the increased resistance does not as a rule disappear. Studying the ability of penicillin-resistant *Staphylococcus aureus* strains to form penicillinase, Spink and Ferris (51) found that staphylococci of two types existed: (a) *Staphylococcus aureus* strains which in vitro grow resistant to penicillin by adaptation. This resistance is not a permanent quality, and these strains do not form penicillinase. (b) The other type becomes resistant in the organism during the treatment. This is a permanent quality. At the same time these strains form penicillinase.

On morphological changes.

With the resistance increasing, it is often found that the bacterial strains form small colonies on agar [Youmans *et al.* (58), Schnitzer *et al.* (49), Eriksen (15)]. Considerable morphological changes are reported by Klimmek *et al.* (35). They

made a *Staphylococcus aureus* strain 60,000 times more resistant to penicillin; the strain had become gram-negative and was capable of anaerobic growth. *Harvey et al.* (27) isolated a *Streptococcus faecalis* strain from blood before and after aureomycin treatment. The resistance to aureomycin of the latter had increased, and they formed smaller colonies of slower growth on agar than the original strain. *Murray et al.* (40) did not find that strains that had acquired resistance to streptomycin in the organism revealed any morphological changes.

On the relationship between virulence and resistance.

McKee and Houck (39) investigated the increased resistance of staphylococci, pneumococci and streptococci to penicillin and in experiments with mice found that increased penicillin resistance reduced the virulence. *Chandler and Schoenbach* (7) investigated the increased resistance of staphylococci, pneumococci and streptococci to streptomycin, without finding that strains which had grown resistant to streptomycin were less virulent than the original strains. They carried out their virulence tests in »fresh human blood«.

Eriksen (16) cultivated hemolytic streptococci in broth containing penicillin. With resistance increasing by less than ten times, a slight reduction of virulence could be discovered in experiments with mice. With resistance increasing up to 20—40-fold the virulence decreased considerably. Was there any change in the virulence of a strain cultivated free of penicillin?

Feldman et al. (18) report that they effected guinea-pig experiments with the strains found in vitro to be resistant to and sensitive to streptomycin, and found that the guinea-pigs that had received streptomycin-sensitive tubercle bacilli died after an average of 70.5 days, whereas the group that had received streptomycin-resistant bacilli lived an average of 95.5 days. This would indicate that the more resistant tubercle strains were less virulent.

Antibiotics as growth factors.

A special group among the bacterial strains that had become resistant is constituted by those that, with the resistance gained, had

become dependent on the antibiotic. Paine and Finland (42) describe strains of *Streptococcus*, *Staphylococcus aureus*, *E. coli*, *Proteus morgani*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* which, while growing resistant to streptomycin also became dependent on this antibiotic for their growth. Compared with the duration and the resistance, they consider the dependence on streptomycin a relatively reversible phenomenon. Pratt and Dufrenoy (44) divide micro-organisms into different classes, according to their reaction to streptomycin. Fig. 1.

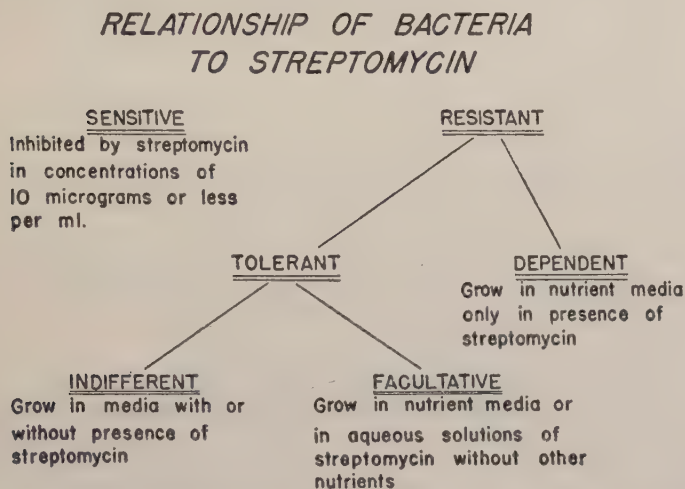


Fig. 1. Diagram to show reactions of different species of bacteria to streptomycin.
(Pratt and Dufrenoy)

On preventing increase in resistance.

It is well-known that the growth of tubercle bacillus depends on the metabolization of para-amino-benzoic acid, and that para-amino-salicylic acid is capable of an inhibitory effect on this process. PAS by itself, therefore, is capable of inhibiting, partly, the growth of tubercle bacillus (44). With streptomycin and PAS in combination, good results have been attained in therapy, and tubercle bacillus strains resistant to streptomycin have at the same time been avoided. An investigation in vitro, interesting in this respect, was carried out by Graessle and Pietrowski (25). By adding small

amounts of para-amino-salicylic acid to the nutrient medium they prevented the *Mycobacterium tuberculosis* strain from becoming resistant to streptomycin. Table 1.

TABLE 1

THE ACTION OF PARA-AMINO-SALICYLIC ACID IN PREVENTING THE DEVELOPMENT OF RESISTANCE TO STREPTOMYCIN.
(GRAESSLE AND PIETROWSKI)

Strain	Drug	Initial sensitivity (concentration per ml)	No. of days exposed	Inhibiting concentration after exposure	
				Streptomycin units per ml	PAS mg per ml
H37Rv	Streptomycin	0.8 units	120	10,000—20,000	0.5
H37Rv	PAS	1.0 mg	120	0.8	1.0
H37Rv	Streptomycin plus PAS	0.6 units			
		0.3 microgram	120	0.8	1.0

Increased resistance to one antibiotic and the resulting simultaneous changes in sensitivity to other antibiotics.

In resistance investigations frequent indications (2, 9, 27, 47, 50) have been found of the increase in resistance to one antibiotic not being accompanied by simultaneous increased resistance to other antibiotics. *Spink et al.* (50) investigated the resistance in vitro of staphylococci to penicillin and sulfonamide, and found no interrelation. *Ranz et al.* (47) found that the streptococci resistant to sulfadiazine (resistance possibly acquired as a result of prophylactic sulfadiazine treatment) were in spite of that sensitive to penicillin. *Demerec* (9), studying the resistance of *Staphylococcus aureus* to streptomycin and penicillin, found that resistance to streptomycin is not accompanied by resistance to penicillin, nor resistance to penicillin by resistance to streptomycin. *Alexander et al.* (2) studied the resistance to streptomycin of various bacterial strains, finding among the strains that were made resistant to streptomycin none that were resistant to polymyxin B, chloromycetin or aureomycin, and they advanced the assumption that the mode of action of polymyxin B, chloromycetin and aureomycin on the bacteria differs from that of streptomycin. *Harvey et al.* (27) isolated a

Streptococcus faecalis strain from blood before and after aureomycin treatment. Although the aureomycin resistance of the latter strain was higher than that of the original strain, the sensitivity of both strains to penicillin remained the same.

Observations of another character have also been effected. Graessle and Frost (24) studied the increase in resistance of *Staphylococcus aureus* strains to penicillin, and carried out simultaneous determinations of sensitivity to streptomycin. They found that, with two strains of *Staphylococcus aureus*, increased resistance to penicillin was accompanied by simultaneous increased resistance to streptomycin. These were the strains with which resistance to penicillin increased most. With three other strains, the sensitivity to streptomycin remained the same. Eisman *et al.* (14) found that two staphylococcus strains, on growing resistant to penicillin G, at the same time became resistant to penicillin X. Similarly, Dowling *et al.* (11) found in their experiments that pneumococcus, streptococcus, staphylococcus and meningococcus strains simultaneously became more resistant to penicillin G and X whenever resistance increased primarily to any one of them. Sullivan *et al.* (52) found that *Staph. aureus*, on becoming more resistant to streptothricin, also displayed increased resistance to streptomycin. Kerner and Morton (34) effected experiments with an *E. coli* strain and found that whenever resistance increased to streptomycin, lavendulin, actinorubin or streptothricin, increased resistance could be observed simultaneously also to all the others. Rake *et al.* (46) found that the increased resistance to streptomycin of one *E. coli* strain was accompanied by a simultaneous increase in resistance to streptomycin B. In their »Antibiotics», published after these investigations, Pratt and Dufrenoy (44) point out, however, that *the increased resistance of bacteria to an antibiotic is characteristic of the antibiotic itself, and does not entail a simultaneous increased resistance to other antibiotics.*

The author's preliminary tests (32), in which the increased resistance of *E. coli* to one antibiotic was found to be accompanied by simultaneous changes in sensitivity to other antibiotics also, were based on observations made in treating a woman of 62 with aureomycin for chronic pyelonephritis (*E. coli*). Following a dosage of 2 g of aureomycin per 24 hours, the urine sample was found to be sterile after 10 days. After 5 days *E. coli* reappeared in the urine.

The sensitivity of this strain to aureomycin (min. inhib. conc. 62.5 $\mu\text{g/ml}$) and chloromycetin (31.25 $\mu\text{g/ml}$) was lower than that of the original strain (aureomycin 15.6 $\mu\text{g/ml}$, chloromycetin 3.9 $\mu\text{g/ml}$), while the sensitivity of the two strains to dihydrostreptomycin was the same (0.97 $\mu\text{g/ml}$). The experiments following this case were carried out with three *E. coli* strains, the resistance of which was raised by subcultivating them in broth in which the dilution of the antibiotic concentration was always doubled. Taking the bacteria always from the first turbid tube in the dilution series, seven transfers were effected, after which sensitivity to the other antibiotics was determined by means of the same dilution series method. The results are given in Tables 2, 3 and 4.

TABLE 2

SENSITIVITY TO AUREOMYCIN, CHLOROMYCETIN AND DIHYDROSTREPTOMYCIN OF THE THREE *E. COLI* STRAINS AFTER SEVEN TRANSFERS IN BROTH CONTAINING AUREOMYCIN. (CONCENTRATIONS INHIBITING VISIBLE GROWTH $\mu\text{g/ml}$.)

	Aureomycin		Chloromycetin		Dihydrostrept.	
	before	after	before	after	before	after
<i>E. coli</i> strain 1	0.97	31.25	3.9	62.5	0.97	0.48
» » » 2	1.9	1.9	3.9	3.9	3.9	0.97
» » » 3	1.9	62.5	15.6	250	15.6	3.9

It is apparent that aureomycin caused a simultaneous increase in resistance to chloromycetin with Strains 1 and 3 (Table 2). The sensitivity of Strain 2 to chloromycetin remained unchanged. Nor did the aureomycin-resistance of the same strain increase. Dihydrostreptomycin sensitivity was not reduced — the opposite tendency could be discerned.

TABLE 3

SENSITIVITY TO CHLOROMYCETIN, AUREOMYCIN AND DIHYDROSTREPTOMYCIN IN THREE *E. COLI* STRAINS AFTER SEVEN TRANSFERS IN BROTH CONTAINING CHLOROMYCETIN. (CONCENTRATIONS INHIBITING VISIBLE GROWTH $\mu\text{g/ml}$.)

	Chloromycetin.		Aureomycin		Dihydrostrept.	
	before	after	before	after	before	after
<i>E. coli</i> strain 1	3.9	250	0.97	3.9	0.97	0.24
» » » 2	3.9	7.8	1.9	3.9	3.9	0.97
» » » 3	15.6	500	1.9	31.25	15.6	7.8

Resistance of all strains to aureomycin (Table 3) increased simultaneously with resistance to chloromycetin. The sensitivity of all strains to dihydrostreptomycin was slightly increased.

TABLE 4

SENSITIVITY TO DIHYDROSTREPTOMYCIN, AUREOMYCIN AND CHLOROMYCETIN IN THREE *E. COLI* STRAINS AFTER SEVEN TRANSFERS IN BROTH CONTAINING DIHYDROSTREPTOMYCIN. (CONCENTRATIONS INHIBITING VISIBLE GROWTH $\mu\text{g/ml.}$)

		Dihydrostrept.		Aureomycin		Chloromycetin	
		before	after	before	after	before	after
<i>E. coli</i> strain	1	0.97	0.97	0.97	0.48	3.9	3.9
» » »	2	3.9	125	1.9	0.48	3.9	0.97
» » »	3	15.6	125	1.9	0.24	15.6	1.9

Sensitivity of all strains to aureomycin (Table 4) was slightly increased. Sensitivity to chloromycetin increased with Strains 2 and 3, but remained unchanged with Strain 1.

From these results it can be seen that increased aureomycin resistance may be accompanied by increased resistance to chloromycetin but increased sensitivity to dihydrostreptomycin. Similarly, increased resistance to chloromycetin may be accompanied by increased resistance to aureomycin and by a correspondingly increased sensitivity to dihydrostreptomycin. Cultivation in broth containing dihydrostreptomycin seemed to be accompanied by somewhat increased sensitivity to chloromycetin and aureomycin. From the results it appears as if some similarity obtains between aureomycin and chloromycetin, as opposed to dihydrostreptomycin, in their mode of action on *E. coli*.

Similar tests were carried out also with terramycin (K a i p a i n e n 33) using *E. coli* as the test organism; the conclusion reached was that terramycin seemed to react to *E. coli* similarly as do aureomycin and chloromycetin. Terramycin also appeared to bear the same relationship to dihydrostreptomycin as that borne by aureomycin and chloromycetin. The author's earlier papers can be summarised in the following observations: (1) *E. coli* cultivation in broth containing aureomycin, chloromycetin or terramycin seemed to be accompanied by increased resistance to each of these three antibiotics, but by somewhat increased sensitivity to dihydrostrepto-

mycin. (2) Cultivation in broth containing dihydrostreptomycin was accompanied by somewhat increased sensitivity to aureomycin, chloromycetin and terramycin.

It was found subsequently that the validity of the author's work reported above is supported by certain findings of Heilmann (30), in which different bacteria were made resistant to an antibiotic by gradually increasing the concentration of this antibiotic in broth, after which sensitivity determinations to other antibiotics were carried out on plates. The results are given in Table 5.

TABLE 5
INDUCED RESISTANCE TO TERRAMYCIN (HEILMAN).

Organism and strain	Made resistant to	Sensitivity in micrograms per milliliter			
		Terra- mycin	Aureo- mycin	Chloram- phenicol	Strepto- mycin
E. coli 687	0	3.12	6.25	6.25	12.5
	Terramycin	100	50	100	6.25
Aerob. aerog. 1,155	0	3.12	3.12	6.25	6.25
	Terramycin	50	50	200	6.25
Strept. faec. 2,323	0	1.56	0.78	12.5	50
	Terramycin	12.5	6.25	12.5	50
E. coli 2,330	0	1.56	3.12	12.5	6.25
	Aureomycin	100	100	100	3.12
Aerob. aerog. 2,319	0	1.56	3.12	6.25	3.12
	Aureomycin	100	50	200	0.78
E. coli 994	0	1.56	3.12	12.5	6.25
	Chloramph.	50	25	200	6.25
Aerob. aerog. 1,155	0	3.12	3.12	6.25	6.25
	Chloramph.	6.25	12.5	200	3.12
E. coli 1,120	0	3.12	3.12	12.5	12.5
	Streptomycin	1.56	1.56	6.25	200
Aerob. aerog. 1,158	0	1.56	1.56	3.12	12.5
	Streptomycin	1.56	0.78	1.56	200

The investigations of Gocke *et al.* (23) differ somewhat from the results of Kaipainen (32, 33) and Heilmann (30). Gocke *et al.* cultivated the different bacteria on agar plates containing rising concentrations of terramycin. At the same time,

original strains were cultivated from day to day without terramycin. Apart from terramycin, the sensitivities of both groups to other antibiotics were determined after 40 transfers. The sensitivities to penicillin, neomycin and chloromycetin were identical in both groups, but a slight increase in the resistance to aureomycin was found with almost all the strains that had been cultivated in nutrient medium containing terramycin. These investigations differ from those of Kaipainen and Heilmann in that the increased resistance to chloromycetin was not acquired simultaneously with the increase in resistance to terramycin. Later on, Pansy *et al.* (43) effected investigations with *E. coli* and *Micrococcus pyog.* strains in which they found that induced resistance to aureomycin was accompanied by resistance to chloromycetin and to terramycin, and that resistance to chloromycetin was accompanied by increased resistance to aureomycin and terramycin. However, the changes with the *Micrococcus pyog.* strain were slighter than those with the *E. coli* strain.

III Subject of Present Investigation

Certain investigations showed that with an increase in resistance to one antibiotic there was a simultaneous increase in resistance to other antibiotics also. This was found to occur, in the first place, among the different forms of one and the same antibiotic, *e. g.* the various penicillins. In addition, simultaneous increase in resistance was also found, in a few cases, to completely different antibiotics, such as streptomycin and penicillin. These results have obviously been phenomena so occasional in character that they have not been capable of reversing the generally held opinion that a simultaneous increase in resistance to two different antibiotics very seldom takes place. As, however, in his preliminary experiments (32, 33), the author made observations which seemed strongly to counter the prevailing conception, it was considered necessary to carry out investigations with more comprehensive material, to find out whether *increase in bacterial resistance to one antibiotic really entails simultaneous changes in sensitivity to other antibiotics as well.*

In addition, it was considered necessary to study the questions related to this subject, and hence the latter part of the investigation included the following:

- The sensitivities to antibiotics of the different individuals of any one bacterial strain may be different (8). Experiments were carried out to compare a parent strain with a more sensitive strain isolated from it, to ascertain whether cultivation in broth containing one antibiotic resulted in different shifts in sensitivity to the other antibiotics also;
- The duration of the induced changes in sensitivity was studied;
- The experiments to study the shifts in sensitivities induced by

antibiotics were carried out with broth as the nutrient medium. Corresponding experiments were effected, also, with other nutrient media;

- The effect of the different antibiotic concentrations and cultivation periods of different lengths on the changes in sensitivity were studied;
- Experiments were made into whether it was possible, by combining the antibiotics, to prevent increase in resistance.

IV Antibiotics, Bacteria and Methods Employed

Antibiotics. When choosing the antibiotics for the present tests, our principal interest was focussed on aureomycin, chloromycetin, terramycin and dihydrostreptomycin, all available in Helsinki. The fact that, even in the earlier investigations, dihydrostreptomycin had been used instead of streptomycin, was due to the fact that streptomycin, in Finland at least, seems to have been almost entirely replaced by dihydrostreptomycin. The preparations used were:

1. **Aureomycin hydrochloride** (Lederle Laboratories Division)
2. **Chloromycetin** (Parke, Davis & Co.)
3. **Terramycin hydrochloride** (Chas. Pfizer & Co.)
4. **Dihydrostreptomycin sulfate** (Parke, Davis & Co.)
5. **Crystalline Penicillin G, sodium salt** (Imperial Chemicals Ltd.)

Commercial antibiotic preparations had to be used in the sensitivity determinations because, in the initial stages of the work, only aureomycin and penicillin were available as standard preparations.

Bacteria. The original experiments were carried out with three *E. coli* strains. The present investigation included, apart from five *E. coli* strains, an additional 22 bacterial strains. The choice was based, in the first place, on the pathogenicity of the bacteria. The bacteria also included three *B. subtilis* strains representing spore-forming species. Although the streptococci employed, when cultivated in broth, produced certain difficulties as regards the reading of the growth results, the bacteria selected, however, constituted a collection possible of cultivation in roughly identical nutrient medium. This was considered necessary so as to ensure the comparability of results. The number of the bacterial strains and species is not very great, but an increase in their number would cause an expansion of the work that would make the comparison of results difficult. All the bacteria used were strains isolated from patients at the Department of Serology and Bacteriology, University of Helsinki, or old strains from the collection. Before taking the bacteria into use, pure cultures were made of them on blood plates for four days. The following strains were employed:

Denomination			
<i>Escherichia coli</i> (2914)	routine 1	<i>E. coli</i> str.	1
— » — (2877)	— » —	— » —	2
— » — (647)	collection 2	— » —	3
— » — (Neapolitana 433)	— » —	— » —	4
— » — (4385)	routine	— » —	5
<i>Aerobacter aerogenes</i> (Mononen)	— » —	<i>A. aerog.</i> str.	1
— » — (2923)	— » —	— » —	2
— » — (4772)	— » —	— » —	3
<i>Salmonella typhi</i> (Kauff. 52)	collection	<i>S. typhi</i> str.	1
— » — (» 62)	— » —	— » —	2
— » — (» 57)	— » —	— » —	3
<i>Proteus vulgaris</i> (3088)	routine	<i>Proteus vulgaris</i> str.	1
— » — (1045)	collection	— » —	2
— » — (2719)	routine	— » —	3
<i>Pseudomonas aeruginosa</i> (20/9)	collection	<i>Ps. aeruginosa</i> str.	1
— » — (4920)	routine	— » —	2
<i>Staphylococcus aureus</i> (1055)	collection	<i>Staph. aureus</i> str.	1
— » — (2968)	routine	— » —	2
— » — (Orion)	collection	— » —	3
— » — (5014)	routine	— » —	4
<i>Staphylococcus albus</i> (5013)	routine	<i>Staph. albus</i> str.	1
<i>Streptococcus pyogenes</i> (Lep.)	collection	<i>Strept. pyog.</i> str.	1
— » — (SF 130)	— » —	— » —	2
— » — (H 8)	— » —	— » —	3
<i>Bacillus subtilis</i> (17 Reinik.)	— » —	<i>B. subtilis</i> str.	1
— » — (12 »)	— » —	— » —	2
— » — (47 »)	— » —	— » —	3

Method. In sensitivity determinations and in daily sub-cultures of bacteria in rising antibiotic concentrations, the serial dilution technic was employed. The nutrient medium used was broth, of which the pH was adjusted to 7.35 (by means of Beckman's pH meter) prior to sterilisation in the autoclave. If, after that, it was found to remain within the limits 7.3—7.4, the broth was accepted. The experiments with each bacterium were carried out with simultaneously prepared broth, which to prevent infection had been divided into small lots to meet the requirements of one day. The broth was inoculated from the pure culture on the blood dish and placed overnight in an incubator. From the overnight culture a dilution 10^{-4} was made the next day into broth. 1 ml of the aqueous solution or suspension, respectively, of the antibiotic, containing e.g. 1,000 $\mu\text{g}/\text{ml}$, was pipetted into the first tube of the series (Wassermann tubes 10×100 mm). Of the bacterial broth mixture, 1 ml was then pipetted into each tube of the series. The contents of the

1 strain taken from routine samples.

2 an old strain taken from collections of the Department.

first tube were mixed carefully, after which, using the same pipette, 1 ml of the mixture was transferred into the second tube, from this 1 ml to the third, and so on. This yielded a serial dilution of the antibiotics in which the concentration was reduced by half in each successive tube. Each tube, hence, contained 1 ml of broth mixture and the antibiotic concentrations were: 500, 250, 125, 62.5 $\mu\text{g/ml}$ and so on. The weakness with a serial dilution made in this way is that the amount of bacteria in the first tubes of the series is less than in the others, and similarly the broth is more diluted. However, both bacterial amount and broth are, from the third tube onwards, almost unchanged in practice. Hence, the concentration of the first tube was made high. Due to the high initial concentration, a small precipitate was formed at the bottom of the first and second tube of the aureomycin and terramycin series. The growth of resistant bacterium in these tubes, however, made it easy to read off the growth result.

Although, in the method employed, the first two tubes of the series were technically not fully comparable with the other tubes of the series, they were nevertheless included in the final results as, with the resistance of the bacteria increasing, they did not indicate growth too readily, and hence did not lead to errors in the final results. Although the initial pH's of the antibiotics employed were different, they did not alter the pH in the first tube of the series more than 0.1. A great advantage of this method is that it is very quick and simple, and pipetting into the same tube is required only twice, which reduces both the risk of infection and errors of pipetting to a minimum. Tubes, one containing broth only, the other the broth-bacterium mixture, served as a control. After the pipetting the tubes were closed with cotton wool plugs and placed in the incubator for 22 hours. Reading off the growth results, the antibiotic concentration of the last clear tube in the series was taken as the sensitivity of the bacterium in question (= the concentration of antibiotics inhibiting visible growth, per ml). To give an increase in the bacterial resistance, bacteria from the first distinctly turbid tube were taken for the next series and a dilution 10^{-2} was made of them, since with a larger amount of inoculum the resistance could be increased more rapidly. In the seventh transfer, however, the dilution 10^{-4} was employed. Increasing the resistance to each bacterial strain was carried out simultaneously with all the antibiotics employed. As a control the original bacterial strain was simultaneously cultivated in broth free of the antibiotic. The sensitivities of this strain too were determined after seven transfers. It was not considered necessary to carry out more than seven transfers using the present method, as it had been ascertained earlier (31) that when the resistance of *E. coli* to aureomycin increased the greatest changes took place during the first 5-6 transfers. This was found to hold good also of the bacteria and antibiotics employed in the present investigation.

The reliability of the serial dilution used was studied with experimental series. The sensitivity of one *E. coli* strain to aureomycin was determined 14 times consecutively. 12 determinations gave the result 15.6 $\mu\text{g/ml}$, and 2 gave 7.8 $\mu\text{g/ml}$. The sensitivity to aureomycin of another *E. coli* strain was shown, in 13 determinations out of 14, to be 3.9 $\mu\text{g/ml}$ and in one determination 7.8 $\mu\text{g/ml}$. The sensitivity to chloromycetin of a third *E. coli* strain proved to be, out of a total of 70 determinations, in 64 of them 15.6 $\mu\text{g/ml}$ and in 6 7.8 $\mu\text{g/ml}$. The deviation, hence, in each case was on the same lines only between two parallel concentrations. *Taking into*

consideration the error yielded by the method, differences exceeding one tube even had to be considered as significant. When carrying out the investigations reported in the latter part of this paper, sensitivity determinations of two *E. coli* strains were effected once again using the standard preparations of antibiotics (with the exception of chloromycetin), and the sensitivities were found to have remained the same as at the beginning, although another broth was used and the laboratory age of the strains in question had increased. In the control series, with seven transfers carried out in broth free of antibiotics, it was found that in 106 cases out of 120 the sensitivity remained constant. In 14 cases a difference of one tube was found.

Table 6 gives the sensitivities of the bacteria employed to the antibiotics used

TABLE 6

ORIGINAL SENSITIVITIES OF THE BACTERIAL STRAINS EMPLOYED IN CONTROL SERIES, WITH BACTERIA CULTIVATED IN BROTH ONLY. THE FINAL SENSITIVITIES WERE THE SAME, EXCEPT IN CASES WHERE THE DEVIATING VALUE IS ENTERED IN BRACKETS.

		Aureo- mycin μg/ml	Chloro- mycetin μg/ml	Terra- mycin μg/ml	Dihydro- strept. μg/ml	Peni- cillin units/ml
<i>E. coli</i>	str. 1	3.9	3.9	3.9	15.6	>62.5
»	» 2	1.9	3.9	3.9	31.25	>62.5
»	» 3	15.6	7.8	0.48	0.24	>62.5
»	» 4	3.9 (1.9)	7.8	1.9 (0.97)	31.25	>62.5
»	» 5	3.9 (1.9)	7.8	3.9	7.8	>62.5
<i>A. aerog.</i>	» 1	15.6	7.8	3.9	0.24	>62.5
»	» 2	62.5	15.6(7.8)	1.9	7.8	>62.5
»	» 3	3.9	7.8	1.9 (0.97)	1.9	>62.5
<i>S. typhi</i>	» 1	1.9	3.9	1.9	62.5	15.6
»	» 2	1.9	3.9	1.9	125	15.6
»	» 3	1.9	3.9	3.9	125	31.25
<i>Proteus vulgaris</i>	» 1	500	7.8	500	31.25	>62.5
»	» 2	250	62.5	500	62.5	>62.5
»	» 3	125	3.9	62.5	7.8	>62.5
<i>Ps. aeruginosa</i>	» 1	62.5	500(250)	31.25	62.5	>62.5
»	» 2	125	250(500)	31.25	>500	>62.5
<i>Staph. aureus</i>	» 1	0.12	3.9	0.12	15.6	0.06
»	» 2	0.48	7.8	0.48	3.9	0.06
»	» 3	0.06	3.9	0.06(0.12)	1.9	0.03
»	» 4	0.12	3.9	0.24	>500	0.24
<i>Staph. albus</i>	» 1	0.24	1.9	0.24	0.97	0.0076
<i>Strept. pyog.</i>	» 1	0.24	7.8	0.48(0.24)	7.8	0.12
»	» 2	0.48 (0.97)	7.8	0.48	15.6	0.0038
»	» 3	0.06	1.9	0.24(0.48)	7.8	0.0076(0.0038)
<i>B. subtilis</i>	» 1	1.9	3.9(7.8)	7.8	250	0.06
»	» 2	3.9	3.9	15.6	7.8	0.03
»	» 3	0.12 (0.24)	7.8	0.12	31.25	0.06

in the investigation. When the sensitivity of the bacterium was, or at any stage of the investigation had risen over 500 $\mu\text{g/ml}$, the top limit was not titrated, but the reading $> 500 \mu\text{g/ml}$ was systematically entered. As to penicillin, in corresponding cases, the entry > 62.5 units/ml was used, which is so high that values exceeding it are of no practical significance.

After raising the resistance of the bacteria to one primary antibiotic, the possible simultaneous changes in sensitivity to other antibiotics were determined. In this so-called cross testing certain principles were observed when judging the partial turbidities possibly present at the growth limit in the serial dilution. If the clear tubes (no growth) of the serial dilution are denoted $-$, slightly turbid $(+)$ and turbid $++$ (distinct visible growth), the following three serial dilutions, for instance, were obtained:

No. of tube	1	2	3	4	5	6	7	8	9	10
Conc. $\mu\text{g/ml}$	500	250	125	62.5	31.25	15.6	7.8	3.9	1.9	0.97
1.	$-$	$-$	$-$	$-$	$-$	$(+)$	$+$	$+$	$+$	$+$
2.	$-$	$-$	$(+)$	$+$	$+$	$+$	$+$	$+$	$+$	$+$
3.	$-$	$-$	$-$	$-$	$-$	$-$	$-$	$-$	$(+)$	$+$

1. Sensitivity of parent strain 31.25 $\mu\text{g/ml}$.
2. Sensitivity determination of the same strain in which the resistance is found to have increased from 31.25 $\mu\text{g/ml} \rightarrow 125 \mu\text{g/ml}$. When reading off the growth result, no too high value was obtained for the increase in resistance.
3. Sensitivity determination of the same strain, in which it is found that the resistance has decreased from 31.25 $\mu\text{g/ml} \rightarrow 3.9 \mu\text{g/ml}$.

Although the mode of action of the antibiotics *in vivo* is different from that *in vitro*, is it necessary to take into consideration, in estimating the resistance of bacteria to an antibiotic, the concentration level attained in the organism when the antibiotic in question is administered according to the so-called normal dosage.

In giving the results of the present experiments the author has endeavoured to indicate, in Tables 7–31, the average concentration levels of the antibiotics in the blood. This is indicated by the vertical broken line running at the level the author has accepted as appropriate for each antibiotic. This line indicating the average concentration of aureomycin, chloromycetin and terramycin has been drawn on the basis of Welch's (55) investigations. If 0.50 g of aureomycin is administered every sixth hour, the serum concentration is 1 $\mu\text{g/ml}$ –4 $\mu\text{g/ml}$. With a similar dosage the serum concentration of terramycin is 2 $\mu\text{g/ml}$ –4 $\mu\text{g/ml}$. The average concentration level of both aureomycin and terramycin has been taken as between 1.9 $\mu\text{g/ml}$ and 3.9 $\mu\text{g/ml}$. With the same dosage again, the serum concentration of chloromycetin is over 4 $\mu\text{g/ml}$ but under 7 $\mu\text{g/ml}$. The line indicating the average concentration level has been drawn between 3.9 $\mu\text{g/ml}$ and 7.8 $\mu\text{g/ml}$.

Buggs *et al.* (5) found that when 500,000 units of streptomycin were administered *i.v.* or *i.m.* every sixth hour, the blood concentration was so high that it prevented the growth of bacteria with a sensitivity to streptomycin of between 5 and 7.5 units. Levin *et al.* (37) found that the streptomycin and dihydrostreptomycin concentrations

present in the organism were of the same value. The line indicating the average concentration level of dihydrostreptomycin has been drawn between 3.9 $\mu\text{g/ml}$ and 7.8 $\mu\text{g/ml}$.

Frisk (22) gave 300,000 units of procaine penicillin suspended in oil with 2 % aluminium stearate as an intramuscular injection, and as a result of 15 experiments he drew a curve which showed that the penicillin concentration of serum for 12 hours exceeded 0.2 unit/ml, and that the concentration after 24 hours still exceeded 0.1 unit/ml. On this basis the line indicating the average concentration level has been drawn between 0.24 unit/ml and 0.12 unit/ml.

V Results of Present Investigation

A. CHANGES IN SENSITIVITY INDUCED BY CULTIVATION OF BACTERIA IN BROTH CONTAINING THE DIFFERENT ANTIBIOTICS

1. CULTIVATION IN BROTH CONTAINING AUREOMYCIN

Sensitivities of Bacteria to Aureomycin after Cultivation in Broth Containing Aureomycin (Table 7)

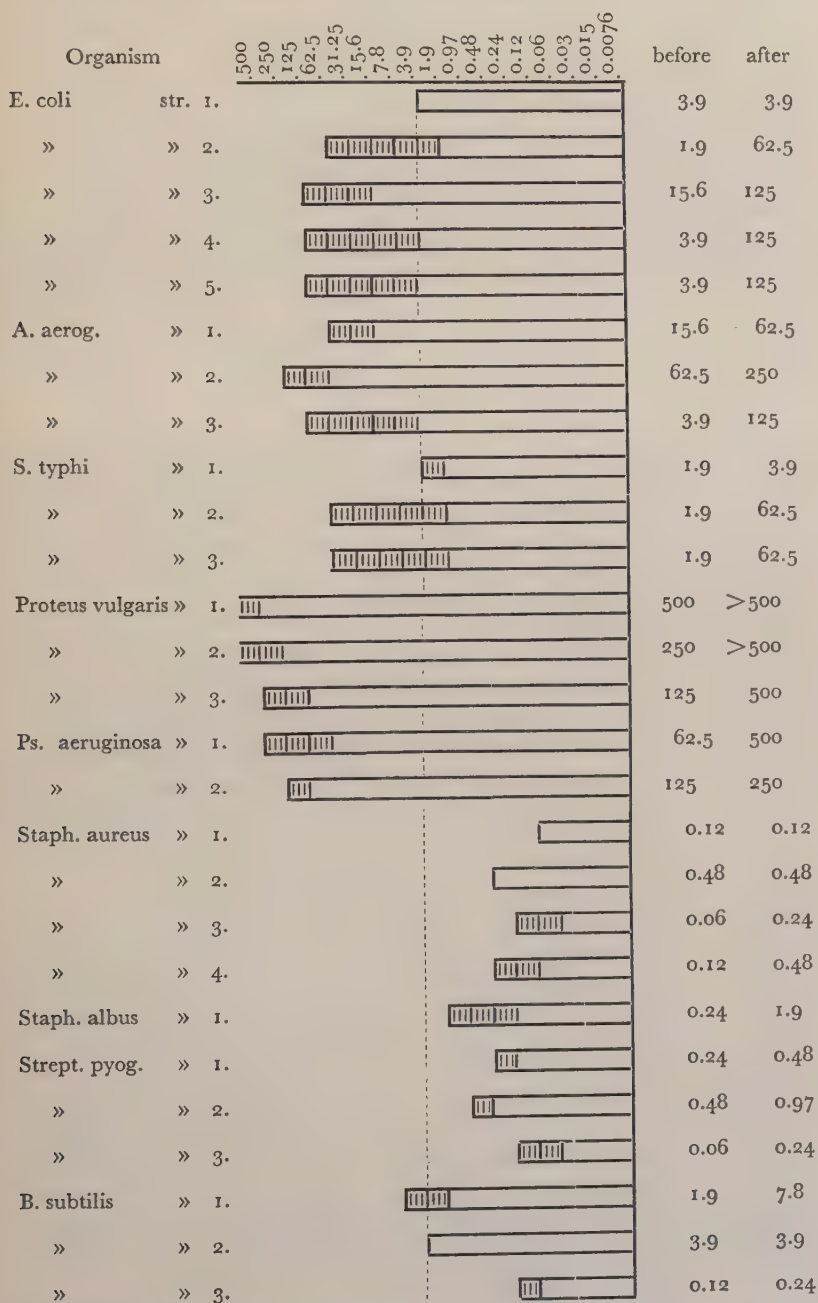
Reviewing the initial sensitivities of bacteria to aureomycin reveals that staphylococci and streptococci strains were more sensitive than the others, and fairly distinctly below the average concentration level. The sensitivities of *E. coli* and *A. aerogenes* strains were above the average level in three cases. This is a point worth considering as it is the bacteria of the coli-aerogenes group that are reckoned to come within the sphere of action of aureomycin. The sensitivities of *S. typhi* strains were just below the average concentration level, while *Proteus vulgaris* and *Ps. aeruginosa* strains were distinctly resistant to aureomycin.

Subjected to aureomycin in increasing doses in broth, the sensitivities of the bacteria, as a rule, decreased somewhat. With *E. coli* strain 1 the sensitivity remained constant throughout, whereas the resistance of the other *E. coli* strains increased and distinctly passed the average concentration level. The increase in resistance of *A. aerogenes* strains was of the same proportion as that of *E. coli* strains. The resistance of *S. typhi* strains 2 and 3 increased distinctly. The resistance of *Proteus vulgaris* and *Ps. aeruginosa*, originally resistant to aureomycin, gained in strength. With two *Proteus vulgaris* strains

TABLE 7

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THE SENSITIVITY OF DIFFERENT BACTERIA TO AUREOMYCIN AFTER SEVEN TRANSFERS IN BROTH CONTAINING AUREOMYCIN.
(MIN. INHIB. CONC. $\mu\text{g/ml.}$)



The broken line indicates the average concentration level of aureomycin in the blood caused by normal dosage.

the final resistance exceeded 500 $\mu\text{g/ml}$. The sensitivities of staphylococci and streptococci were somewhat reduced but remained below the average concentration level. Price *et al.* (45) succeeded in making the resistance of a *Streptococcus haemol.* strain rise 66.6 times in the course of 14 transfers, but the sensitivity of their *Staph. aureus* strain remained at the original 0.08 $\mu\text{g/ml}$., which corresponds to the sensitivities to aureomycin of our *Staphylococcus aureus* strains employed in the present investigations, the sensitivities of strains 1 and 2 showing no decrease either. The sensitivity of *B. subtilis* strains decreased to the same degree as that of staphylococci.

Sensitivities of Bacteria to Chloromycetin after Cultivation in Broth Containing Aureomycin (Table 8)

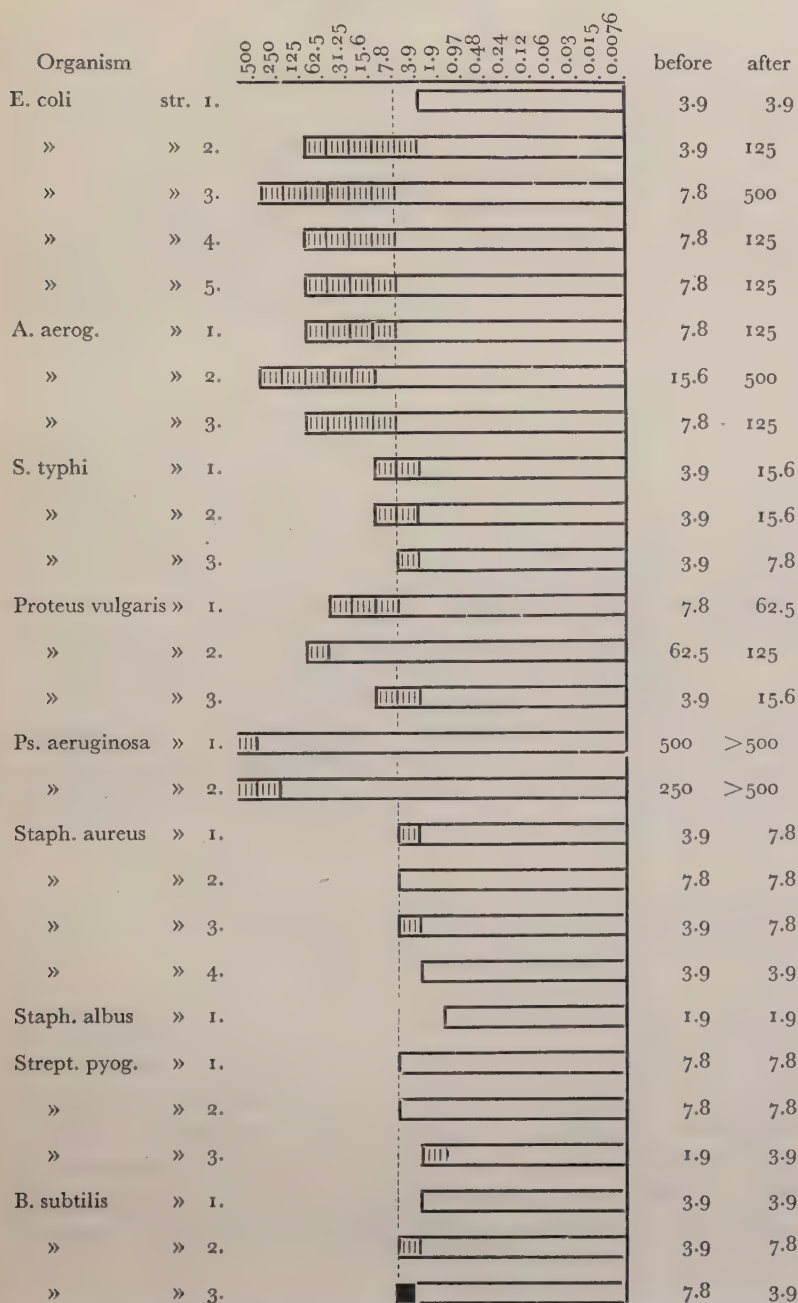
In investigations by Kaipainen (32) and Heilmann (30) it was found that the increased resistance of *E. coli* to aureomycin was accompanied by an increase in resistance to chloromycetin also. *E. coli* strain 1, of which the resistance to aureomycin did not increase when grown in broth containing aureomycin, seemed at the same time to have retained its original sensitivity to chloromycetin. In earlier experiments by Kaipainen an *E. coli* strain was also found which retained its sensitivity in a similar test. However, the resistance of *E. coli* strains 2—5 increased distinctly, and corresponded to the results attained in previous experiments by Kaipainen and by Heilmann. The resistance of each of the three *A. aerogenes* strains increased by 16—32 times, the increase being of the same range as with *E. coli* strains 2—5. The sensitivity of *S. typhi* strains was slightly reduced, the sensitivity of two of them exceeding the average concentration level. The resistance of *Proteus vulgaris* strains 1 and 3, fairly sensitive to chloromycetin, also increased above the average concentration level. The originally resistant *Ps. aeruginosa* strains became more resistant still. No changes worth mentioning took place in the sensitivities of the staphylococci, streptococci and *B. subtilis* strains.

The general impression is that cultivation in broth containing aureomycin resulted in increased resistance to chloromycetin with *E. coli*, *A. aerog.*, *S. typhi*, *Proteus vulgaris* and *Ps. aeruginosa* strains, whereas the sensitivities of staphylococci, streptococci and *B. subtilis* remained unchanged.

TABLE 8

33

THE SENSITIVITY OF DIFFERENT BACTERIA TO CHLOROMYCETIN AFTER SEVEN TRANSFERS IN BROTH CONTAINING AUREOMYCIN.
(MIN. INHIB. CONC. $\mu\text{g/ml.}$)



The broken line indicates the average concentration level of chloromycetin in the blood caused by normal dosage.

Sensitivities of Bacteria to Terramycin after Cultivation in Broth Containing Aureomycin (Table 9)

In Heilmann's (30) experiments the resistance of *E. coli* to terramycin increased after cultivation in aureomycin from 1.56 $\mu\text{g/ml}$ to 100 $\mu\text{g/ml}$. From Table 9 it can be seen that the sensitivity of *E. coli* strain 1 remained unchanged, whereas the resistance of the other *E. coli* strains to terramycin increased considerably. The initial sensitivity of *E. coli* strain 2 was 3.9 $\mu\text{g/ml}$, and the final sensitivity 500 $\mu\text{g/ml}$. With *E. coli* strains 2–5 the final sensitivity rose distinctly above the average concentration level. Similarly, the resistance of all *A. aerog.* strains increased above the average concentration level. The resistance of *S. typhi* strains increased less, and their ultimate sensitivity was slightly above the average concentration level. The resistance of the *Proteus vulgaris* and *Ps. aeruginosa* strains, originally resistant to terramycin, increased further. The staphylococci and streptococci retained their initial sensitivity, and no reduction in sensitivity worth mentioning was observed. The sensitivities of *B. subtilis* strains 1 and 3 decreased slightly.

The changes resulting from cultivation in broth containing aureomycin in the sensitivities to terramycin and to chloromycin were on the same lines. The staphylococci and streptococci, in general, retained their original sensitivity. The changes proper took place with *E. coli*, *A. aerog.*, *S. typhi*, *Proteus vulgaris* and *Ps. aeruginosa* strains, and all the changes were on the same lines.

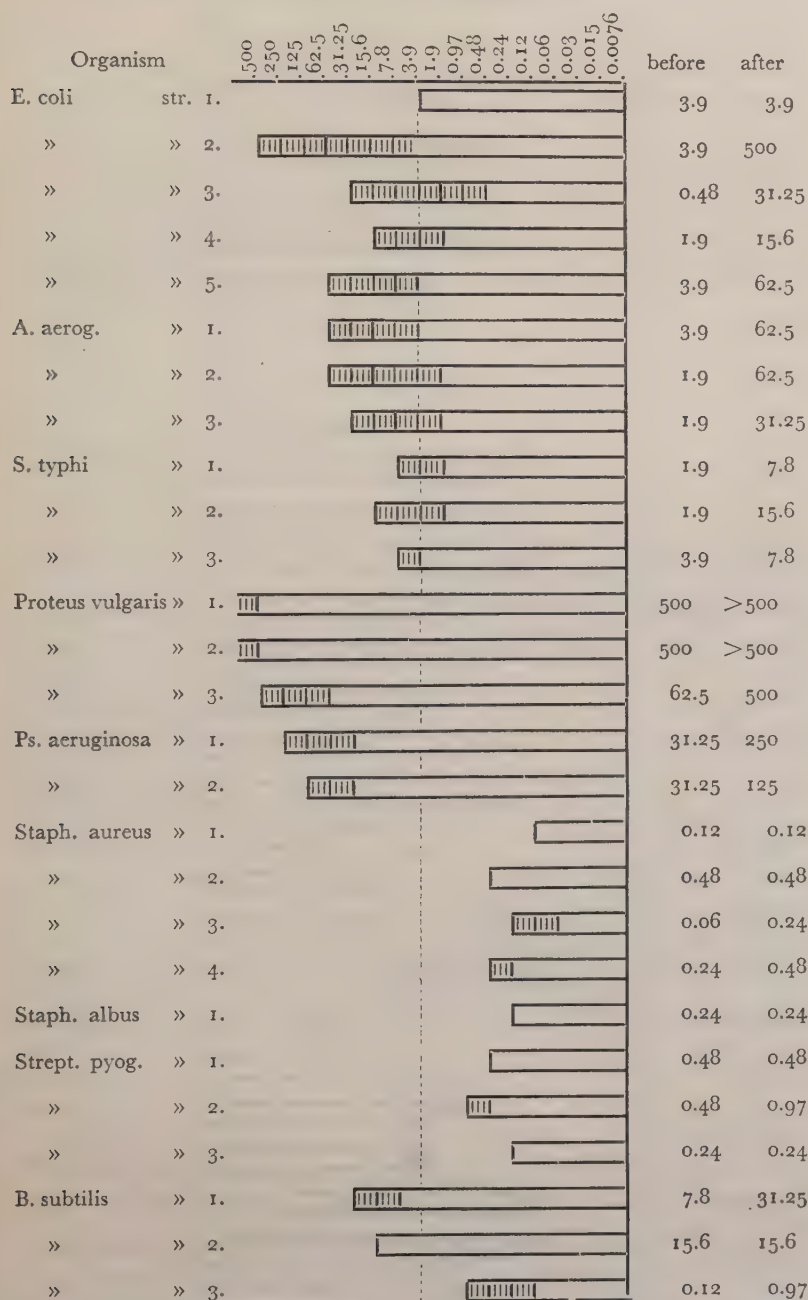
Sensitivities of Bacteria to Dihydrostreptomycin after Cultivation in Broth Containing Aureomycin (Table 10)

In Kaipainen's (32) investigations the sensitivities of *E. coli* strains to dihydrostreptomycin increased after cultivation in broth containing aureomycin, independent of whether their resistance to aureomycin increased or not. The increase in sensitivity was slight, but nevertheless worthy of note. In Heilmann's (30) investigations the sensitivities to streptomycin of *E. coli* and *A. aerog.* strains increased in the same proportions as in Kaipainen's investigations.

TABLE 9

THE SENSITIVITY OF DIFFERENT BACTERIA TO TERRAMYCIN AFTER SEVEN TRANSFERS IN BROTH CONTAINING AUREOMYCIN.

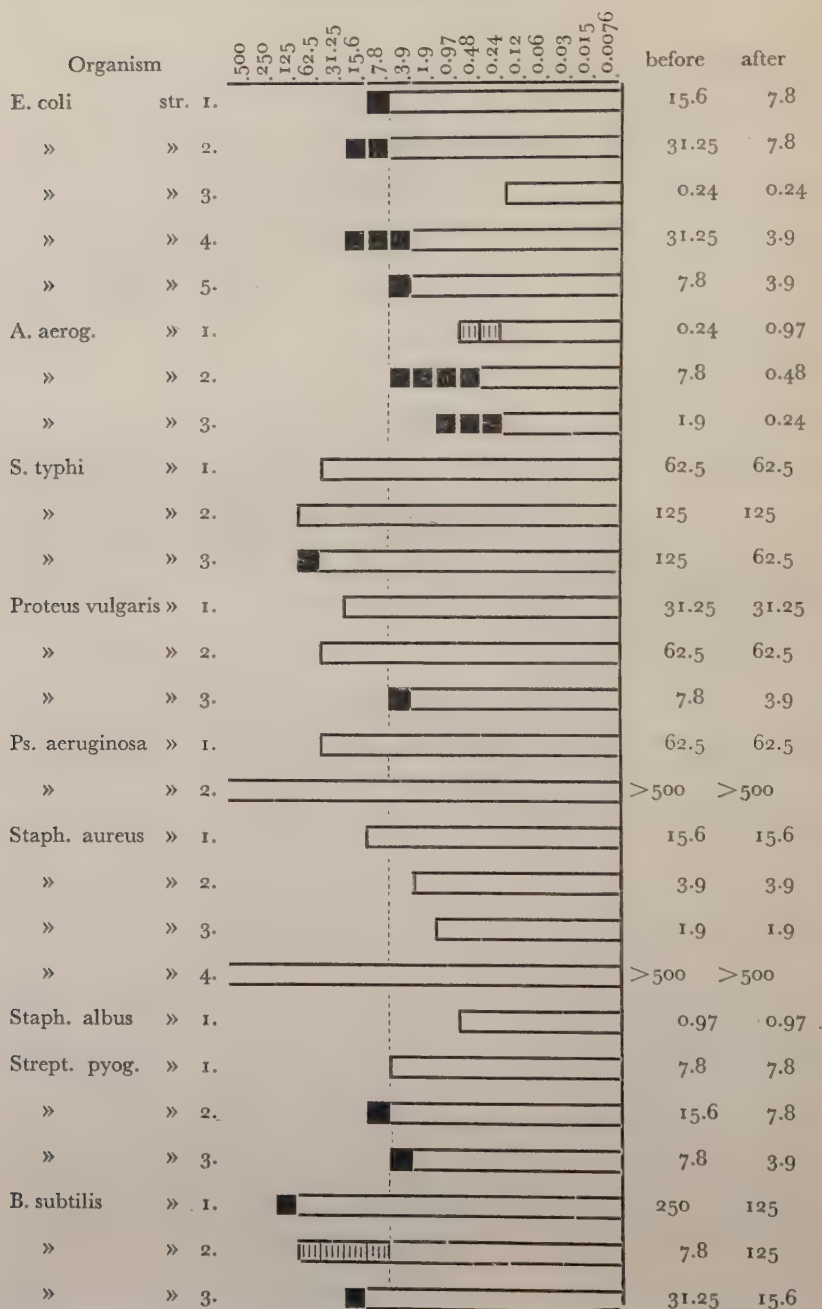
(MIN. INHIB. CONC. $\mu\text{g/ml.}$)



The broken line indicates the average concentration level of terramycin in the blood caused by normal dosage.

TABLE 10

THE SENSITIVITY OF DIFFERENT BACTERIA TO DIHYDROSTREPTOMYCIN AFTER SEVEN TRANSFERS IN BROTH CONTAINING AUREOMYCIN.
(MIN. INHIB. CONC. $\mu\text{g/ml.}$)



The broken line indicates the average concentration level of dihydrostreptomycin in the blood caused by normal dosage.

A glance at Table 10 shows that as a result of cultivation in broth containing aureomycin, changes in sensitivity to dihydrostreptomycin were different from those to chloromycetin and terramycin. In many instances the sensitivity remained unchanged, and in the instances of increased sensitivity the increase was slight. The sensitivity of *E. coli* strain 4 increased distinctly. That of *A. aerog.* strain 1 was reduced although the sensitivity of strains 2 and 3 increased distinctly, and their sensitivities definitely moved farther from the average concentration level. The resistance of *S. typhi*, *Proteus v.* and *Ps. aeruginosa* strains remained almost constant. The sensitivity of the slightly more sensitive staphylococci and streptococci did not undergo any greater change either. *B. subtilis* Strain 2 gave a surprising result in that its resistance increased.

Sensitivities of Bacteria to Penicillin after Cultivation in Broth Containing Aureomycin (Table 11)

It has not been previously established that cultivation in nutrient medium containing aureomycin changes the sensitivity of bacteria to penicillin. In fact, as mentioned earlier (51), resistance to penicillin has been found to increase with the continuous cultivation of staphylococci in broth.

The general impression from Table 11 is that the sensitivities to penicillin changed but slightly. In part, this was perhaps due to the fact that the sensitivities of the *E. coli*, *A. aerog.*, *Proteus v.* and *Ps. aeruginosa* strains resistant to penicillin were not titrated in detail when over 62.5 units/ml.

The sensitivity of *Streptococcus pyog.* strain 1 declined somewhat. The resistance to penicillin of *B. subtilis* strain 3 increased.

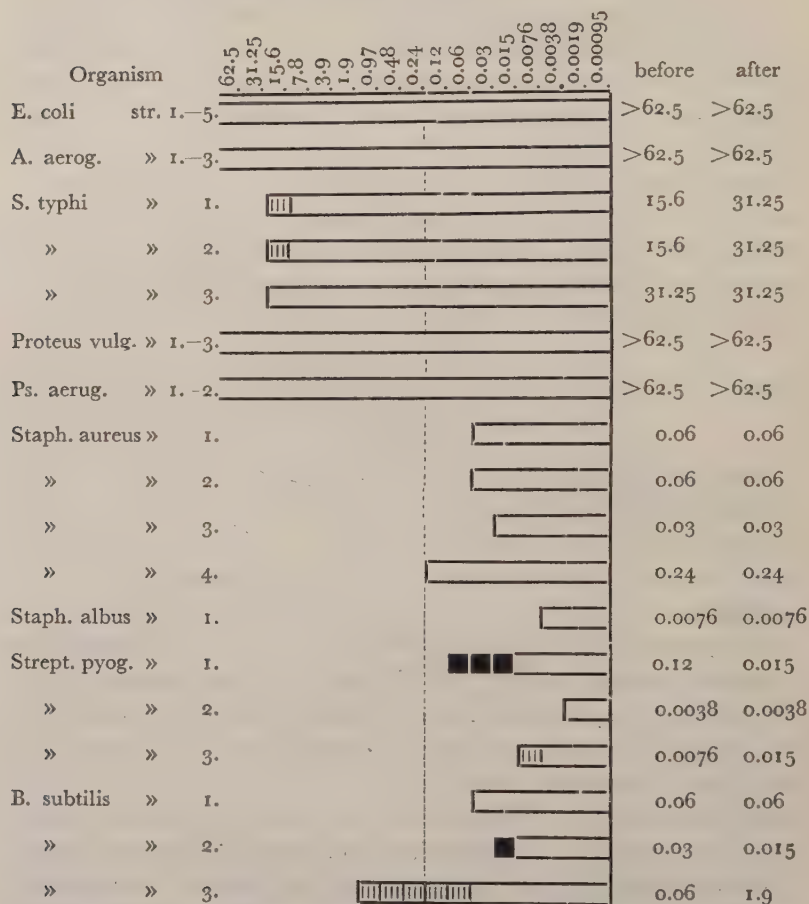
Although the sensitivities to penicillin were found to have changed with a couple of strains after cultivation in broth containing aureomycin, the changes in their entirety were slight and uncorrelated.

Summary

Cultivation of bacteria in broth containing aureomycin induced the following changes in sensitivity to the other antibiotics:

1. The sensitivities to chloromycetin and terramycin seemed to decline with *E. coli*, *A. aerog.*, *S. typhi*, *Proteus v.* and *Ps. aeruginosa* strains. The sensitivities of staphylococcus, strepto-

TABLE 11
THE SENSITIVITY OF DIFFERENT BACTERIA TO PENICILLIN AFTER SEVEN
TRANSFERS IN BROTH CONTAINING AUREOMYCIN.
(MIN. INHIB. CONC. units/ml.)



The broken line indicates the average concentration level of penicillin in the blood caused by normal dosage.

coccus and *B. subtilis* strains showed changes to some extent parallel.

- The sensitivities to dihydrostreptomycin generally remained unchanged or increased slightly with certain *E. coli* and *A. aerog.* strains.
- No changes worth mentioning could be noticed in the sensitivities to penicillin.

2. CULTIVATION IN BROTH CONTAINING CHLOROMYCETIN

Sensitivities of Bacteria to Chloromycetin after Cultivation in Broth Containing Chloromycetin (Table 12)

Reviewing the initial sensitivities of bacteria to chloromycetin revealed that although the average concentration level of chloromycetin is higher than that of aureomycin, the sensitivities broadly speaking remained very close to the average concentration level. Initial chloromycetin sensitivities, with regard to staphylococci and streptococci, differed distinctly from the initial aureomycin sensitivities. A striking feature is that the *Proteus vulgaris* strains were less resistant to chloromycetin than to aureomycin. *Proteus vulgaris* strains 1 and 3 could even be considered sensitive to chloromycetin. *Ps. aeruginosa* strains seemed to be rather resistant to chloromycetin.

When bacteria were cultivated in broth with rising chloromycetin concentrations their sensitivities to chloromycetin generally declined. A comparison with the changes caused by aureomycin, Table 7, shows that chloromycetin resistance increased somewhat more than aureomycin resistance.

E. coli strain 1, of which the resistance to aureomycin did not increase, was more labile in its sensitivity to chloromycetin. Only the resistance to aureomycin of *Proteus vulgaris* strains 1 and 2 (Table 7) exceeded 500 $\mu\text{g/ml}$, but with chloromycetin the 500 $\mu\text{g/ml}$ limit was passed by *E. coli* strains 3 and 4, *A. aerog.* strains 1 and 2, *Proteus vulgaris* strains 1 and 2 and the two *Ps. aeruginosa* strains. The resistance of the staphylococci and the streptococci also revealed a rising tendency. The resistance of *B. subtilis* strains did not increase.

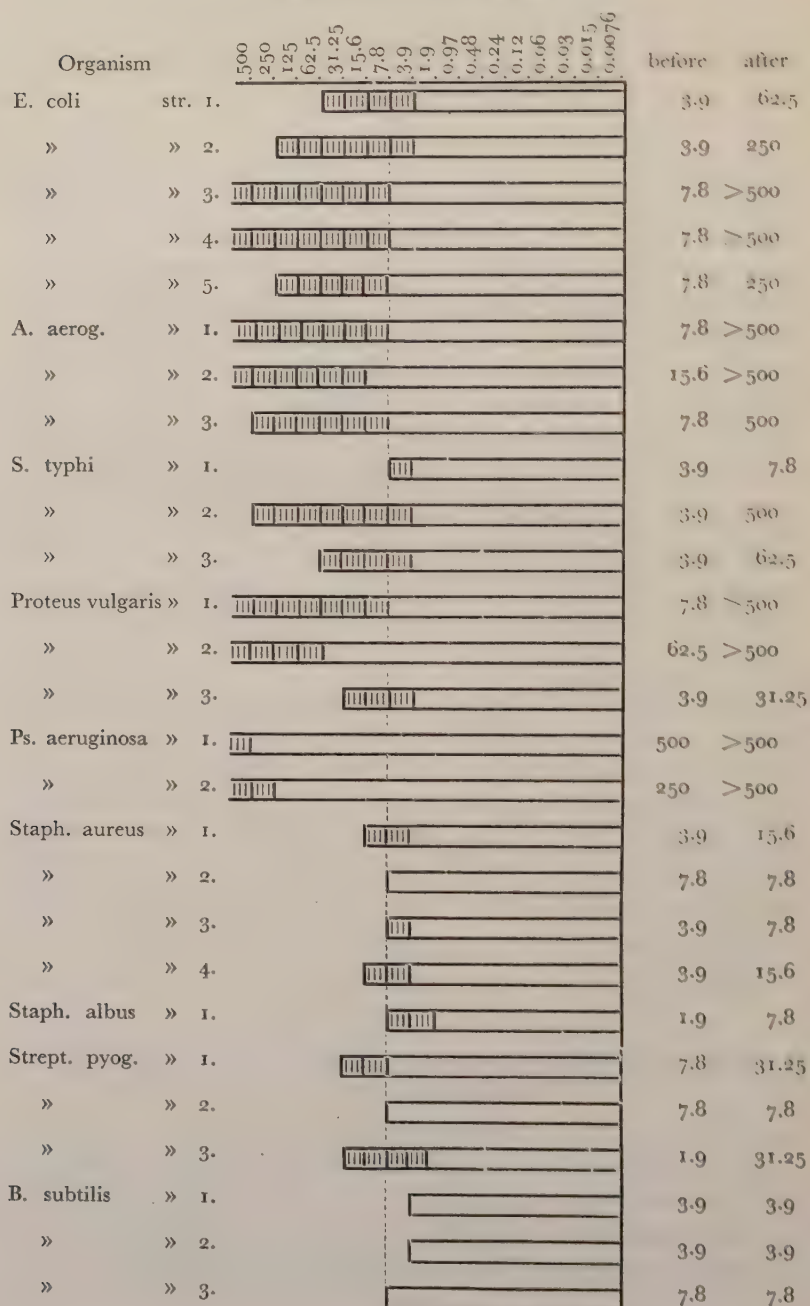
Sensitivities of Bacteria to Aureomycin after Cultivation in Broth Containing Chloromycetin (Table 13)

It was found in Kaipainen's (32) and Heilman's (30) investigations that the increased resistance to chloromycetin of *E. coli* was accompanied by an increased resistance to aureomycin. A review of the results in Table 13 shows that cultivation in broth

TABLE 12

THE SENSITIVITY OF DIFFERENT BACTERIA TO CHLOROMYCETIN AFTER SEVEN TRANSFERS IN BROTH CONTAINING CHLOROMYCETIN.

(MIN. INHIB. CONC. $\mu\text{g/ml.}$)



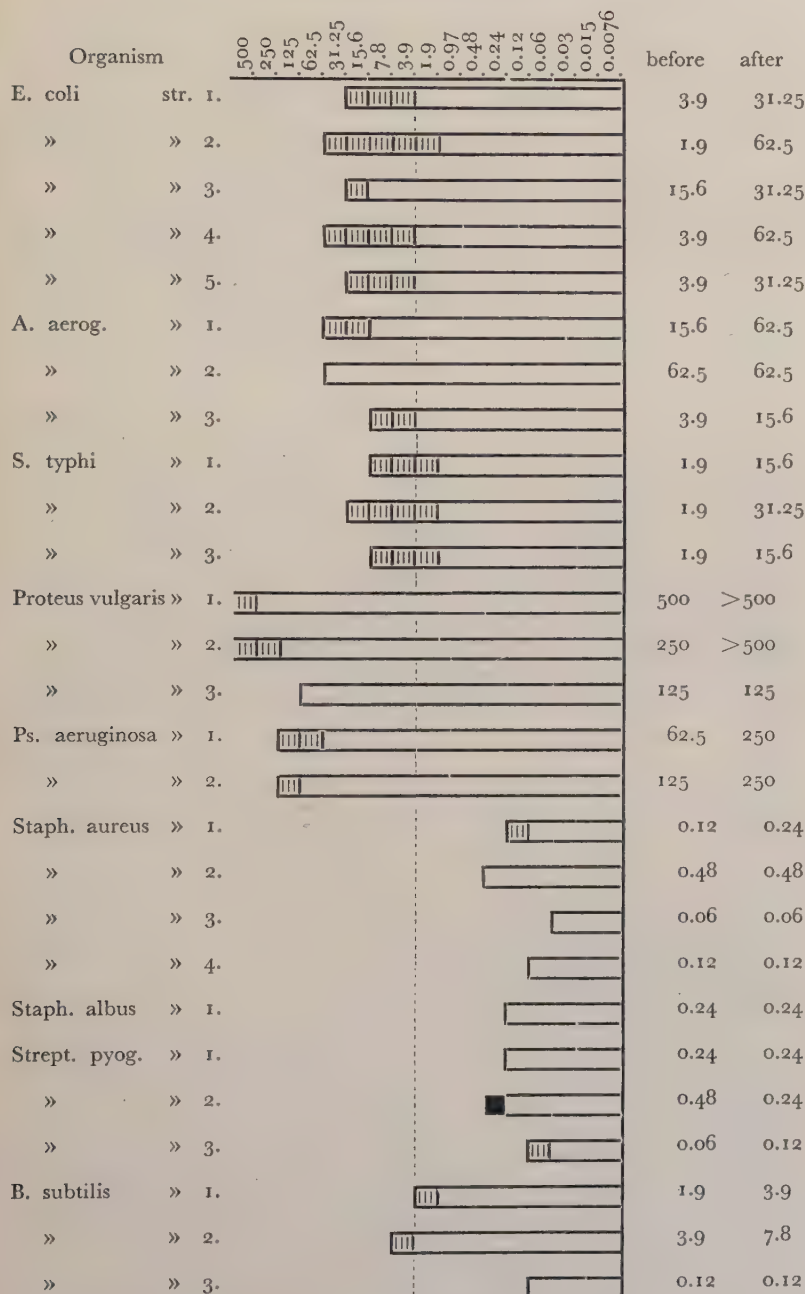
The broken line indicates the average concentration level of chloromycetin in the blood caused by normal dosage.

TABLE 13

41

THE SENSITIVITY OF DIFFERENT BACTERIA TO AUREOMYCIN AFTER SEVEN TRANSFERS IN BROTH CONTAINING CHLOROMYCETIN.

(MIN. INHIB. CONC. $\mu\text{g/ml.}$)



The broken line indicates the average concentration level of aureomycin in the blood caused by normal dosage.

containing chloromycetin induced with some bacteria an increase in resistance to aureomycin.

However, *E. coli* strain 1, of which the resistance to aureomycin did not increase on cultivation in broth containing aureomycin, after cultivation in broth containing chloromycetin acquired increased resistance to aureomycin! With *E. coli* strains 2, 4 and 5, the resistance to aureomycin increased approximately the same as that of strain 1. The resistance of *A. aerog.* strain 2 to aureomycin showed no increase, whereas that of *A. aerog.* strains 1 and 3 grew 4-fold. The resistance to aureomycin of all *S. typhi* strains increased above the average concentration level. With a part of the *Proteus vulgaris* and *Ps. aeruginosa* strains the resistance to aureomycin also revealed a rising tendency.

There were no evident changes in the sensitivities of the staphylococci, streptococci and *B. subtilis* strains.

The general impression is that increased resistance to chloromycetin resulting from cultivation in broth containing chloromycetin was accompanied by a simultaneous increased resistance to aureomycin in a number of *E. coli*, *A. aerog.*, *S. typhi*, *Proteus vulgaris* and *Ps. aeruginosa* strains, but that this resistance was perhaps somewhat slighter in degree than the increase in resistance to chloromycetin accompanying increased resistance to aureomycin (Table 8).

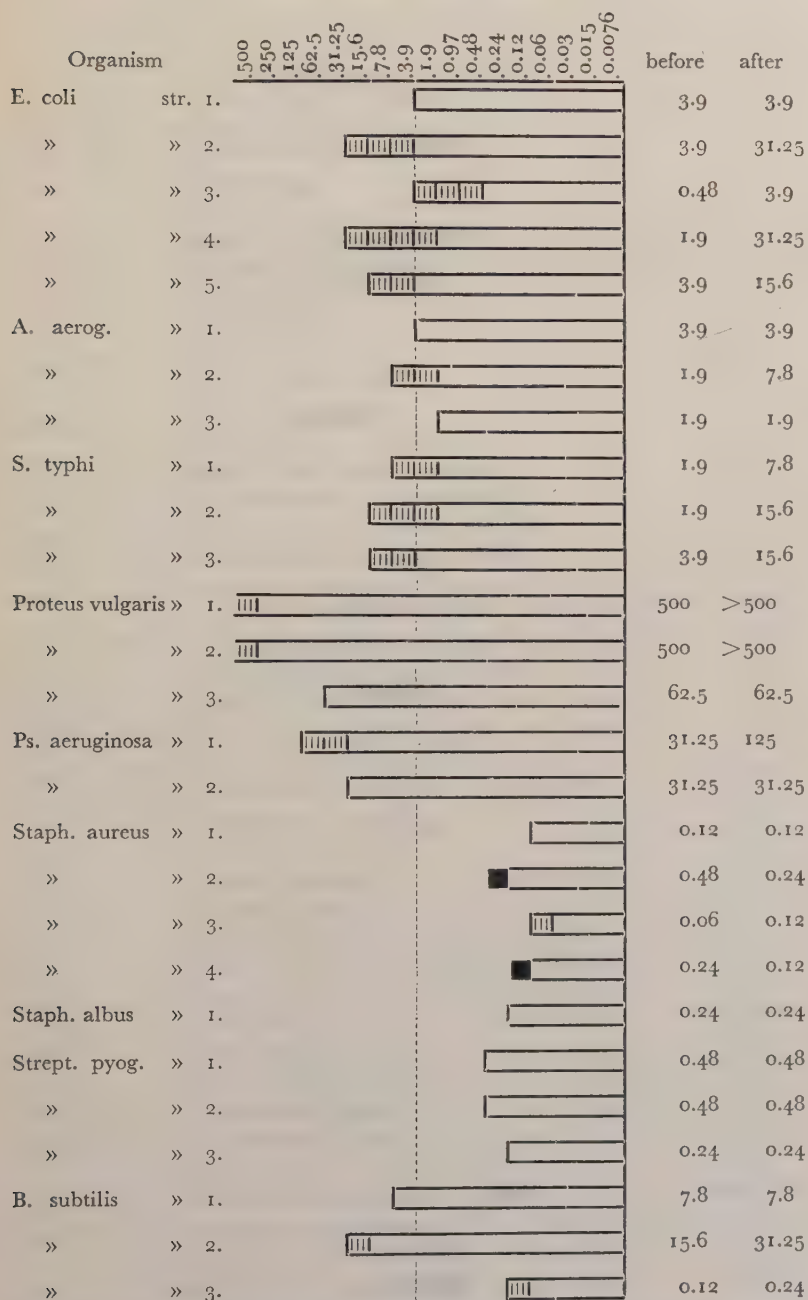
Sensitivities of Bacteria to Terramycin after Cultivation in Broth Containing Chloromycetin (Table 14)

In the experiments by Heilmann (30) resistance to terramycin increased, with an *E. coli* strain, from 1.56 $\mu\text{g/ml}$ $\rightarrow$ 50 $\mu\text{g/ml}$, upon its being made resistant to chloromycetin. Table 14 shows that in our experiments the resistance to terramycin of *E. coli* strains increased by approximately the same proportions as their resistance to aureomycin in Table 13. The resistance of *E. coli* strain 1 to terramycin did not increase, nor did the terramycin resistance of the same strain increase when it was cultivated in broth containing aureomycin (Table 9). The resistance to terramycin of *E. coli* strains 2—5 and a number of *A. aerog.*, *S. typhi*, and *Ps. aeruginosa* strains revealed slight increases.

TABLE 14

THE SENSITIVITY OF DIFFERENT BACTERIA TO TERRAMYCIN AFTER SEVEN TRANSFERS IN BROTH CONTAINING CHLOROMYCETIN.

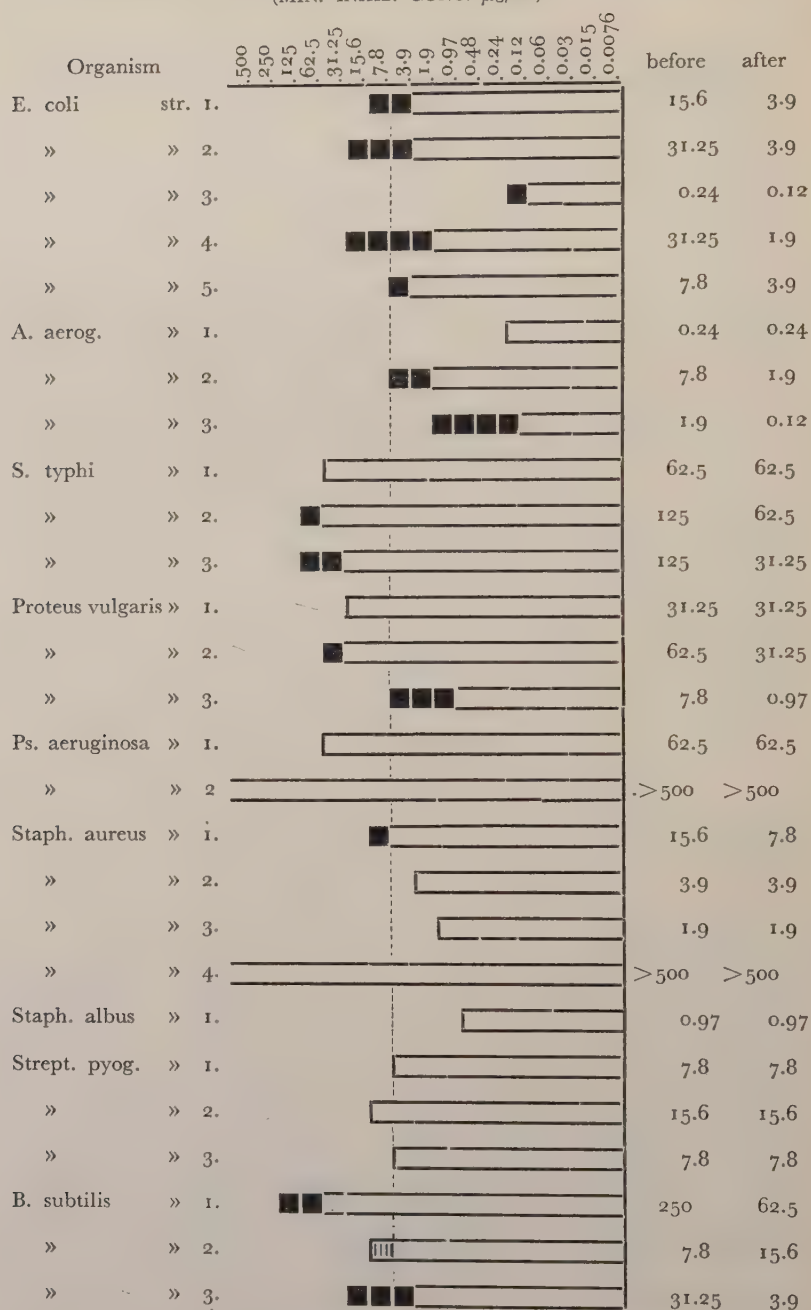
(MIN. INHIB. CONC. $\mu\text{g/ml.}$)



The broken line indicates the average concentration level of terramycin in the blood caused by normal dosage.

TABLE 15

THE SENSITIVITY OF DIFFERENT BACTERIA TO DIHYDROSTREPTOMYCIN AFTER SEVEN TRANSFERS IN BROTH CONTAINING CHLOROMYCETIN.
(MIN. INHIB. CONC. $\mu\text{g/ml.}$)



The broken line indicates the average concentration level of dihydrostreptomycin in the blood caused by normal dosage.

There was no general change in the sensitivities of staphylococcus, streptococcus and *B. subtilis* strains, and one *Proteus vulgaris* strain (the resistance of the other two fell outside the scale).

A comparison of the increases in resistance to terramycin resulting from cultivation in broth containing aureomycin (Table 9) with those resulting from cultivation in broth containing chloromycetin, shows that the latter were slighter.

Sensitivities of Bacteria to Dihydrostreptomycin after Cultivation in Broth Containing Chloromycetin (Table 15)

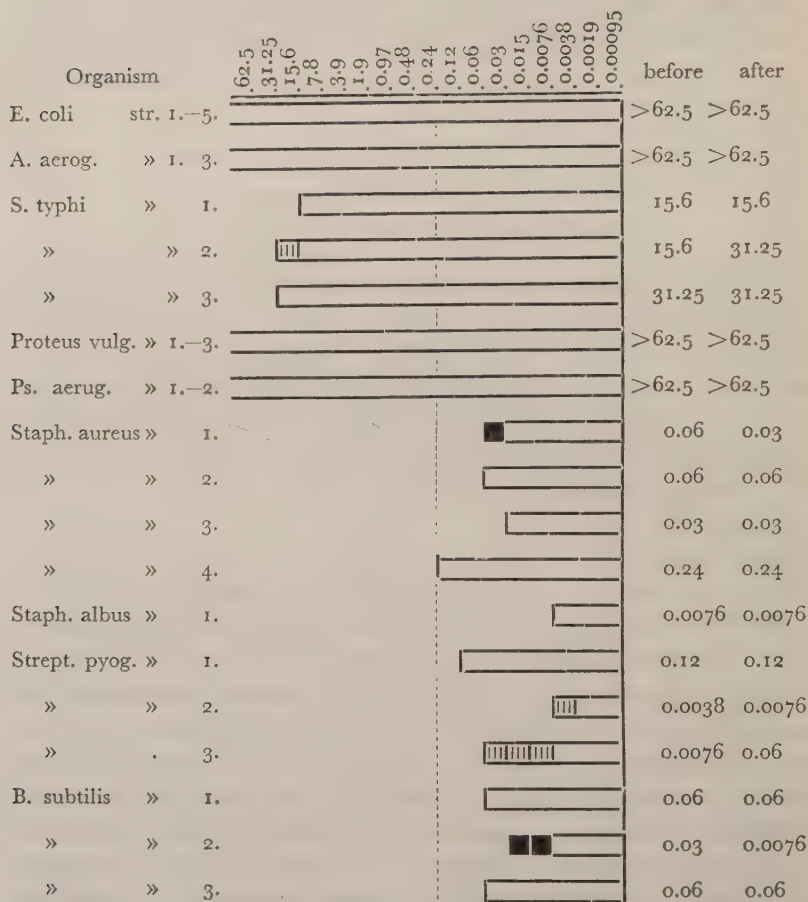
In previous investigations by Kaipainen (32) it appeared that when three *E. coli* strains were cultivated in broth containing chloromycetin, increased resistance to chloromycetin was accompanied by a slightly increased sensitivity to dihydrostreptomycin. In the corresponding experiments by Heilmann (30) *E. coli* sensitivity to streptomycin remained constant, but the sensitivity of an *A. aerog.* strain to streptomycin increased from 6.25 $\mu\text{g/ml}$ $\rightarrow$ 3.12 $\mu\text{g/ml}$.

The results given in Table 15 are similar, as far as *E. coli* is concerned, to those obtained in the author's previous investigations (32). The sensitivity to dihydrostreptomycin of *A. aerog.* strain 1 did not increase, but that of *A. aerog.* strain 3 increased from 1.9 $\mu\text{g/ml}$ $\rightarrow$ 0.12 $\mu\text{g/ml}$. The resistance to dihydrostreptomycin of the same strain declined in nearly the same proportions when cultivated in broth containing aureomycin (Table 10). In a number of *S. typhi* and *Proteus vulgaris* strains sensitivity was somewhat increased. Dihydrostreptomycin resistance of *Ps. aeruginosa* strains remained unchanged, as was also the case when grown in broth containing aureomycin. The sensitivities to dihydrostreptomycin of staphylococcus and streptococcus strains remained unchanged; the same result was achieved after cultivation in broth containing aureomycin. The sensitivities of *B. subtilis* strains 1 and 3 increased.

Hence, cultivation in broth containing chloromycetin resulted in unchanged or slightly increased sensitivity to dihydrostreptomycin with certain *E. coli*, *A. aerog.*, *S. typhi*, *Proteus vulgaris*, and *B. subtilis* strains.

TABLE 16

THE SENSITIVITY OF DIFFERENT BACTERIA TO PENICILLIN AFTER SEVEN TRANSFERS IN BROTH CONTAINING CHLOROMYCETIN.
(MIN. INHIB. CONC. units/ml.)



The broken line indicates the average concentration level of penicillin in the blood caused by normal dosage.

Sensitivities of Bacteria to Penicillin after Cultivation in Broth Containing Chloromycin (Table 16)

Increased resistance to aureomycin was not followed by increased resistance to penicillin. The changes in the sensitivities to penicillin resulting from the cultivation of bacteria in broth containing chloromycin, shown in Table 16, were equally slight.

The increased resistance of *Streptococcus pyog.* strain 3 and the increased sensitivity of *B. subtilis* strain 2 are of hardly any significance.

Summary

Cultivation of bacteria in broth containing chloromycetin induced the following changes in sensitivities to the other antibiotics:

1. Sensitivities to aureomycin and terramycin seemed to decline with some *E. coli*, *A. aerog.*, *S. typhi*, *Proteus vulgaris* and *Ps. aeruginosa* strains.
2. Sensitivities to dihydrostreptomycin remained unchanged or increased slightly, especially with some Gram-negative rods.
3. No general changes could be observed in sensitivities to penicillin.

3. CULTIVATION IN BROTH CONTAINING TERRAMYCIN

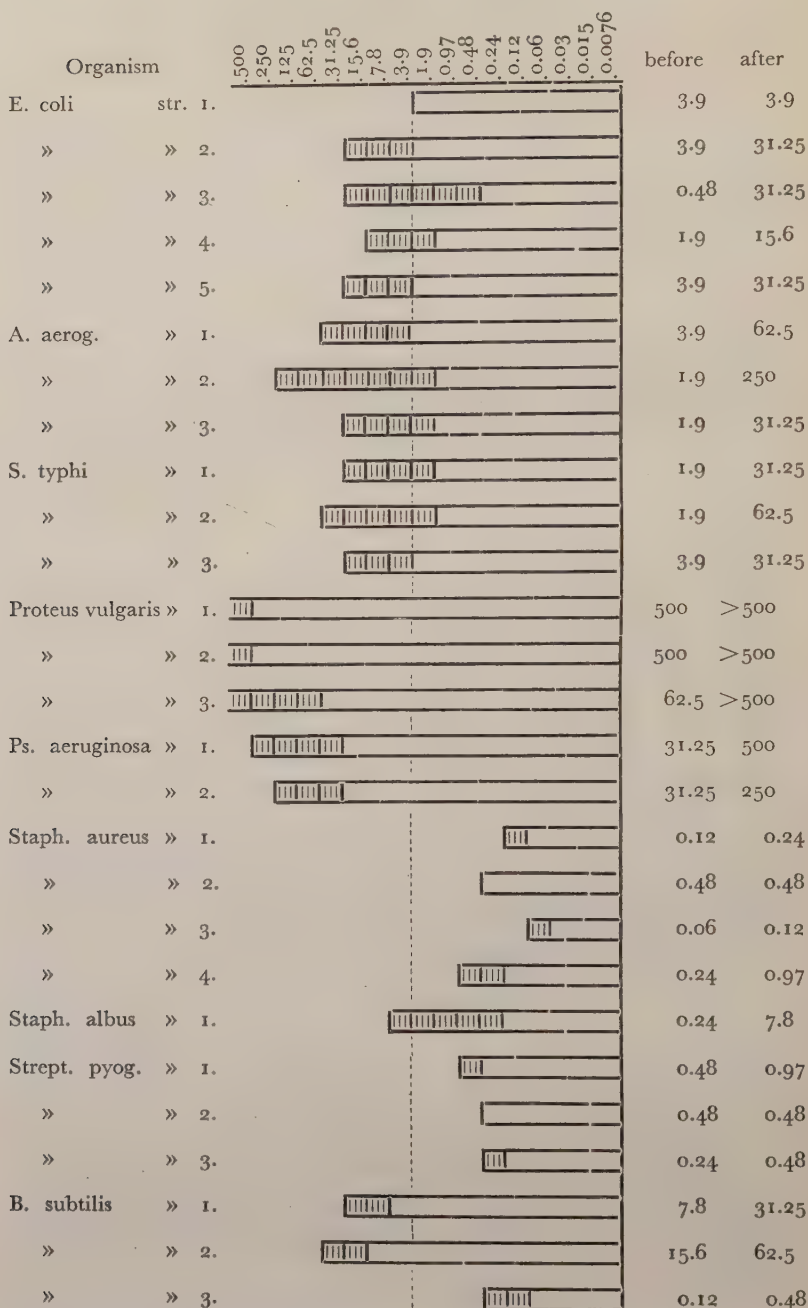
Sensitivities of Bacteria to Terramycin after Cultivation in Broth Containing Terramycin (Table 17)

It is known (55) that the antibiotic spectrum of terramycin covers, broadly speaking, the same bacterial species as that of aureomycin and chloromycetin. Of the three, aureomycin and terramycin are probably more closely connected with one another than with chloromycetin. A comparison of the initial sensitivities to terramycin and aureomycin of the bacteria employed in the present investigation reveals a considerable similarity. The sensitivities of the *E. coli* strains coincided in part. *A. aerog.* strains showed a somewhat greater sensitivity to terramycin than to aureomycin. With *S. typhi* strains, the sensitivities to terramycin and aureomycin were approximately the same. The same relationship obtained with *Proteus vulgaris* and *Ps. aeruginosa* strains, which were resistant both to terramycin and aureomycin. The sensitivities to terramycin and aureomycin of the terramycin-sensitive staphylococcus and streptococcus strains were, by strains even, of approximately the same extent. The *B. subtilis* strains were slightly more resistant to terramycin.

TABLE 17

THE SENSITIVITY OF DIFFERENT BACTERIA TO TERRAMYCIN AFTER SEVEN TRANSFERS IN BROTH CONTAINING TERRAMYCIN.

(MIN. INHIB. CONC. $\mu\text{g/ml.}$)



The broken line indicates the average concentration level of terramycin in the blood caused by normal dosage.

After cultivation in broth containing terramycin the sensitivities to terramycin declined in general. The resistance to terramycin of *E. coli* strain 1 did not increase. Nor did the resistance to aureomycin of the same strain increase after cultivation in broth containing aureomycin. Resistance to terramycin of the other *E. coli* strains increased approximately in the same proportions as to aureomycin after corresponding action (Table 7). The same relationship also obtained with *S. typhi*, *Proteus vulgaris*, *Ps. aeruginosa*, staphylococcus, streptococcus and *B. subtilis* strains.

The increased resistance of bacteria to terramycin after cultivation in broth containing terramycin, hence, occurred in approximately the same proportions as their increased resistance to aureomycin after cultivation in aureomycin. This increase in primary resistance to aureomycin and terramycin seemed less marked than the increase in resistance to chloromycetin after cultivation in broth containing chloromycetin.

Sensitivities of Bacteria to Aureomycin after Cultivation in Broth Containing Terramycin (Table 18)

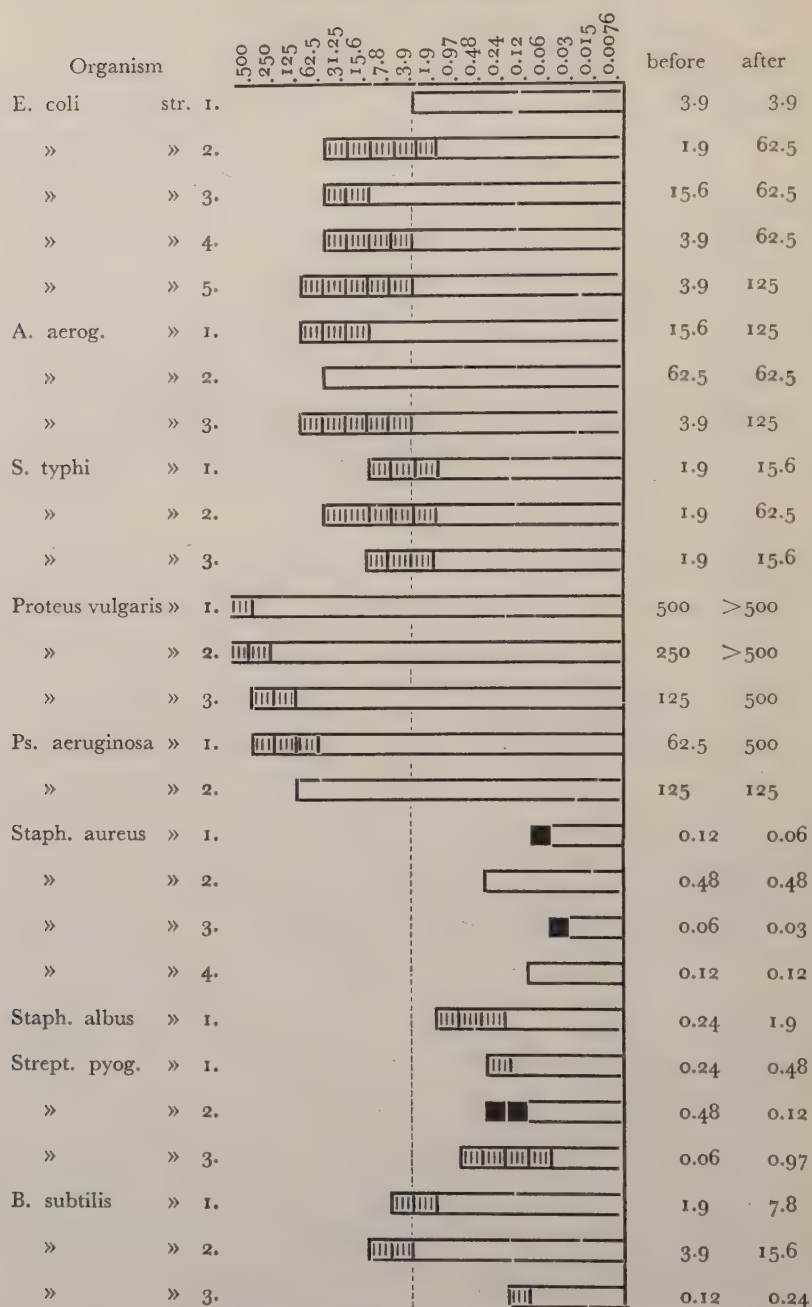
In the investigations by Heilmann (30) increased resistance to terramycin was accompanied by an increased resistance to aureomycin with *E. coli*, *A. aerog.* and *Strept. faecalis* strains. Similarly, in investigations by Gocke *et al.* (23) the increased resistance to terramycin was accompanied by increased resistance to aureomycin with almost all the strains employed by them.

The general impression from Table 18 is that cultivation in broth containing terramycin resulted in increased resistance to aureomycin. The increase in the resistance of *E. coli* and *A. aerog.* strains was of approximately the same extent as in Heilmann's investigations. In addition, with both an *E. coli* and an *A. aerog.* strain there was no shift in sensitivity. The resistance of the *S. typhi* strains increased in the same proportions as that of the *E. coli* strains. The increase in the resistance of *E. coli*, *A. aerog.* and *S. typhi* strains resulted in the final sensitivities exceeding the average concentration level.

The *Proteus vulgaris* strains initially resistant to aureomycin acquired increasing resistance to aureomycin. The same applies to *Ps. aeruginosa* strain 1. On the other hand, the sensitivity of Strain

TABLE 18

THE SENSITIVITY OF DIFFERENT BACTERIA TO AUREOMYCIN AFTER SEVEN TRANSFERS IN BROTH CONTAINING TERRAMYCIN.
(MIN. INHIB. CONC. $\mu\text{g/ml.}$)



The broken line indicates the average concentration level of aureomycin in the blood caused by normal dosage.

2 remained unchanged. The sensitivity of *Staphylococcus aureus* strains did not decline. On the other hand, the resistance of the *Staph. albus* strain and *Strept. pyog.* strain 3 showed a distinct increase, whereas the sensitivity of *Strept. pyog.* strain 2 revealed signs of increasing. The resistance of *B. subtilis* strains 1 and 2 seemed to increase slightly.

The general impression received is that cultivation in broth containing terramycin resulted in increased resistance to aureomycin with *E. coli*, *A. aerog.*, *S. typhi*, *Proteus vulgaris* and *Ps. aeruginosa* strains. A similar increase was found with the *Staph. albus*, one *Strept. pyog.* strain and two *B. subtilis* strains.

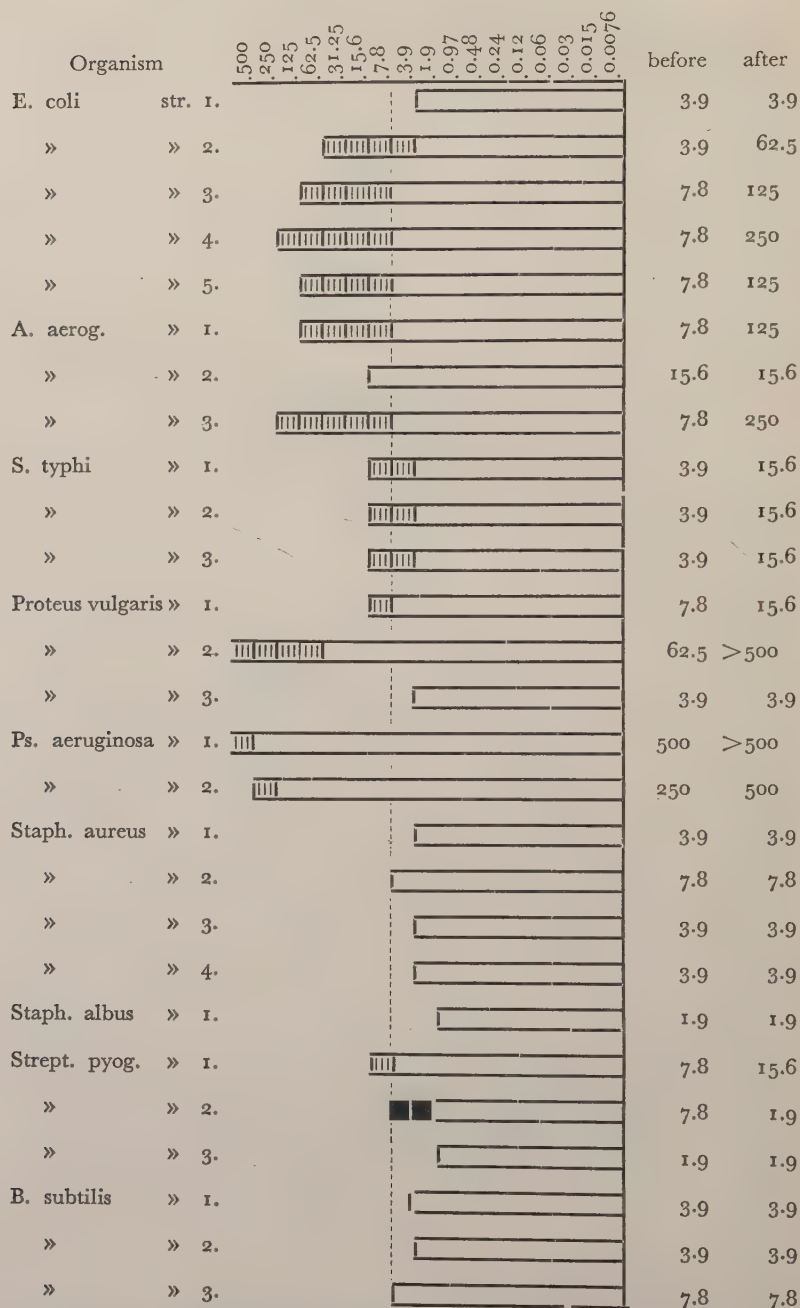
Sensitivities of Bacteria to Chloromycetin after Cultivation in Broth Containing Terramycin (Table 19)

In investigations by Gocke *et al.* (23) increased resistance to terramycin was accompanied by increased resistance to aureomycin, but not to chloromycetin. On the other hand, in Heilmann's (30) experiments, increased resistance to terramycin was accompanied by increased resistance both to aureomycin and to chloromycetin with one *E. coli* strain and with one *A. aerog.* strain. The resistance to chloromycetin of Heilmann's *E. coli* strain increased from 6.25 $\mu\text{g/ml}$ $\rightarrow$ 100 $\mu\text{g/ml}$. The changes in sensitivity of our *E. coli* strains given in Table 19 were approximately of the same proportions. *E. coli* strain 1 constituted an exception, there being no rise in the resistance. The sensitivities of *A. aerog.* strains declined in the same proportions as the sensitivities to chloromycetin of *E. coli* strains. The sensitivities of *S. typhi* strains decreased slightly, and were above the average concentration level. The resistance of *Proteus vulgaris* strain 2 increased distinctly. The sensitivity to chloromycetin of *Staph. aureus*, *Staph. albus* and *B. subtilis* strains showed no change. The sensitivities of the *Ps. aeruginosa* and *Strept. pyog.* strains also remained almost unchanged.

Cultivation in broth containing terramycin led to the acquisition of increased resistance by *E. coli*, *A. aerog.*, *S. typhi* and *Proteus vulgaris* strains. The sensitivities of *Ps. aeruginosa*, *staphylococcus*, *streptococcus* and *B. subtilis* strains remained almost unchanged.

TABLE 19

THE SENSITIVITY OF DIFFERENT BACTERIA TO CHLOROMYCETIN AFTER SEVEN TRANSFERS IN BROTH CONTAINING TERRAMYCIN.
(MIN. INHIB. CONC. $\mu\text{g/ml.}$)



The broken line indicates the average concentration level of chloromycetin in the blood caused by normal dosage.

Sensitivities of Bacteria to Dihydrostreptomycin after Cultivation in Broth Containing Terramycin (Table 20)

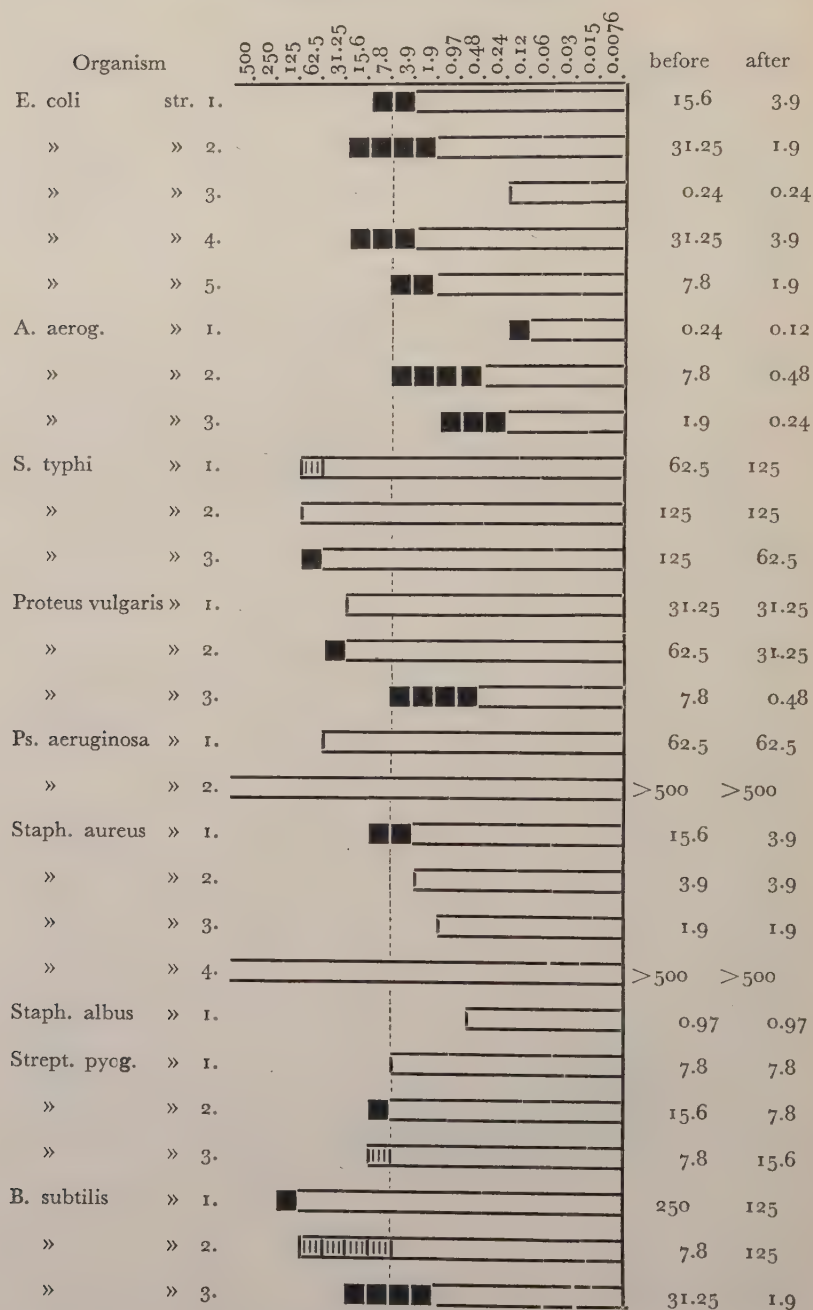
The resistance to dihydrostreptomycin revealed a declining tendency after cultivation both in broth containing aureomycin and in broth containing chloromycetin. Similarly, in Heilmann's (30) investigations, the resistance of an *E. coli* strain to streptomycin declined from 12.5 $\mu\text{g/ml}$ $\rightarrow$ 6.25 $\mu\text{g/ml}$. On the other hand, the sensitivity of the *A. aerog.* and *Streptococcus faecalis* strains remained unchanged.

Table 20 reveals the same rising tendency in sensitivity changes as occurred in the sensitivities to dihydrostreptomycin after cultivation in broth containing aureomycin and broth containing chloromycetin. The sensitivity of *E. coli* strain 3 showed no change; the resistance of the other *E. coli* strains decreased slightly. Similarly, *A. aerog.* strains 2 and 3 became distinctly more sensitive. The sensitivities of *S. typhi* strains showed no general change, and the sensitivity of *Proteus vulg.* strain 3 increased distinctly. The sensitivity of *Ps. aeruginosa* strains remained unchanged. The sensitivity of *Staphylococcus aureus* strain 1 increased slightly. With other *Staphylococcus aureus*, *Staph. albus* and *Strept. pyog.* strains there was no general change. The resistance to dihydrostreptomycin of *B. subtilis* strain 2 increased surprisingly, by 16 times. The resistance to dihydrostreptomycin of the same strain also increased after cultivation in broth containing aureomycin.

Hence, cultivation in broth containing terramycin resulted, as a rule, in unchanged or slightly decreased resistance to dihydrostreptomycin. Cultivation both in broth containing aureomycin and in broth containing chloromycetin, similarly, induced slight shifts in the sensitivities to dihydrostreptomycin. The increase in the sensitivity to dihydrostreptomycin resulting from the action of each of the three antibiotics was also of the same extent. *The increase in sensitivity seemed to bear no correlation to the initial sensitivity to dihydrostreptomycin, any more than to whether or not the resistance of the bacteria had increased towards the primary antibiotics (aureomycin, chloromycetin, terramycin).*

TABLE 20

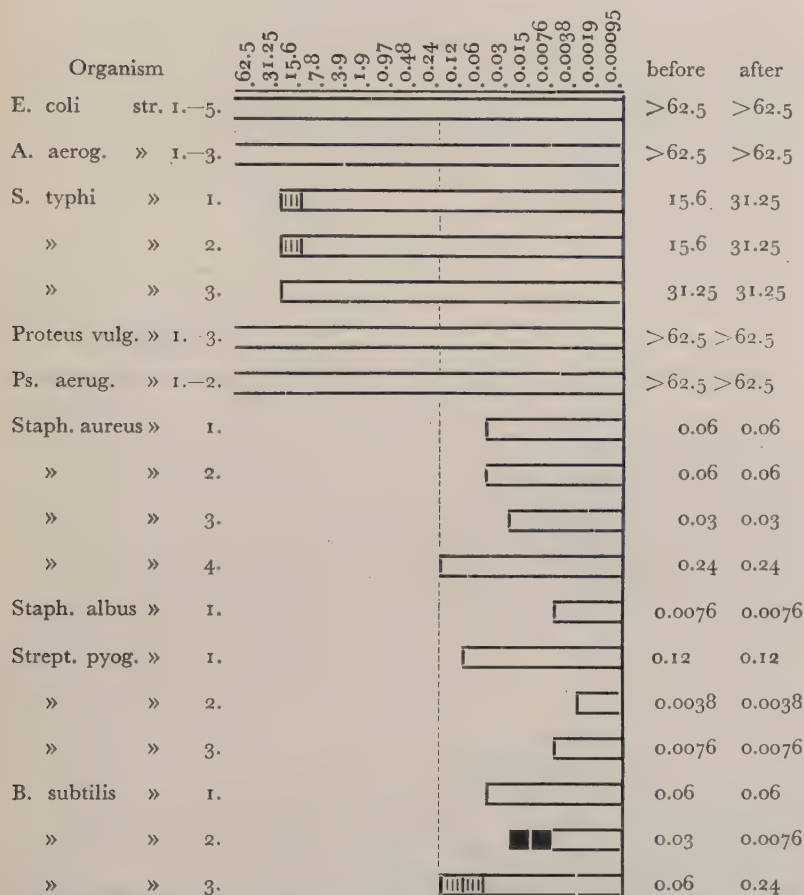
THE SENSITIVITY OF DIFFERENT BACTERIA TO DIHYDROSTREPTOMYCIN AFTER SEVEN TRANSFERS IN BROTH CONTAINING TERRAMYCIN.
(MIN. INHIB. CONC. $\mu\text{g/ml.}$)



The broken line indicates the average concentration level of dihydrostreptomycin in the blood caused by normal dosage.

TABLE 21

THE SENSITIVITY OF DIFFERENT BACTERIA TO PENICILLIN AFTER SEVEN TRANSFERS IN BROTH CONTAINING TERRAMYCIN.
(MIN. INHIB. CONC. units/ml.)



The broken line indicates the average concentration level of penicillin in the blood caused by normal dosage.

Sensitivities of Bacteria to Penicillin after Cultivation in Broth Containing Terramycin (Table 21)

Neither cultivation in broth containing aureomycin nor cultivation in broth containing chloromycetin resulted in altered sensitivities of the bacteria to penicillin. Nor, in Gocke *et al.*'s. (23)

investigations, was increased resistance to terramycin accompanied by sensitivity changes to penicillin.

The changes resulting from cultivation in broth containing terramycin in the sensitivities to penicillin (Table 21) were equally slight as after cultivation in broth containing aureomycin or chloromycetin. The slight changes in sensitivities to penicillin of *B. subtilis* strains 2 and 3, furthermore, contradicted one another.

Hence, cultivation in broth containing terramycin did not result in changed sensitivities to penicillin.

Summary

Cultivation of bacteria in broth containing terramycin induced the following changes in sensitivities to other antibiotics:

1. Sensitivity to aureomycin and chloromycetin seemed to decrease with *E. coli*, *A. aerog.*, *S. typhi*, *Proteus vulgaris* and *Ps. aeruginosa* strains.
2. The sensitivities to dihydrostreptomycin remained unchanged or increased slightly.
3. No general changes were observed in sensitivities to penicillin.

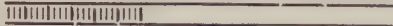
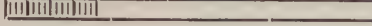
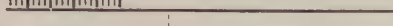
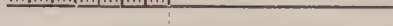
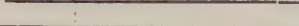
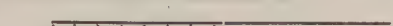
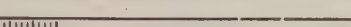
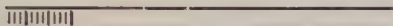
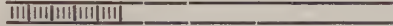
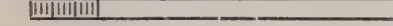
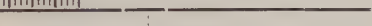
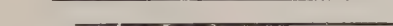
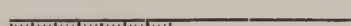
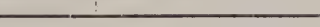
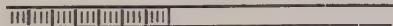
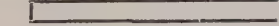
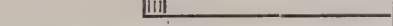
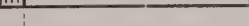
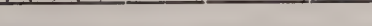
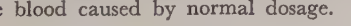
4. CULTIVATION IN BROTH CONTAINING DIHYDROSTREPTOMYCIN

Sensitivities of Bacteria to Dihydrostreptomycin after Cultivation in Broth Containing Dihydrostreptomycin (Table 22)

The increase in bacterial resistance to streptomycin, as is known, is generally very great. Price *et al.* (45) induced an over 226,000-fold increase in the resistance of the *E. typhosa* strain, in the course of 14 transfers in nutrient medium containing streptomycin. The resistance to streptomycin of the *Staph. aureus* strain, in the course of 11 transfers, grew to 83,000-fold. These figures are of a range entirely different from the increase in resistance to the other antibiotics.

From Table 22 it can be seen that the initial sensitivity to dihydrostreptomycin of only a few of the bacterial strains employed in the present investigation was below the average concentration level. Strains with an initial resistance of $>500 \mu\text{g/ml}$ were *Ps.*

THE SENSITIVITY OF DIFFERENT BACTERIA TO DIHYDROSTREPTOMYCIN AFTER SEVEN TRANSFERS IN BROTH CONTAINING DIHYDROSTREPTOMYCIN.
(MIN. INHIB. CONC. $\mu\text{g/ml.}$)

Organism		500	350	250	125	62.5	31.25	15.6	7.8	3.9	1.9	0.97	0.48	0.24	0.12	0.06	0.03	0.015	0.0076	before	after
E. coli	str. 1.																			15.6	>500
	» 2.																			31.25	500
	» 3.																			0.24	31.25
	» 4.																			31.25	>500
	» 5.																			7.8	>500
A. aerog.	» 1.																			0.24	62.5
	» 2.																			7.8	>500
	» 3.																			1.9	250
S. typhi	» 1.																			62.5	>500
	» 2.																			125	>500
	» 3.																			125	>500
Proteus vulgaris	» 1.																			31.25	>500
	» 2.																			62.5	500
	» 3.																			7.8	>500
Ps. aeruginosa	» 1.																			62.5	>500
	» 2.																			>500	>500
Staph. aureus	» 1.																			15.6	250
	» 2.																			3.9	125
	» 3.																			1.9	250
	» 4.																			>500	>500
Staph. albus	» 1.																			0.97	31.25
Strept. pyog.	» 1.																			7.8	>500
	» 2.																			15.6	15.6
	» 3.																			7.8	15.6
B. subtilis	» 1.																			250	500
	» 2.																			7.8	250
	» 3.																			31.25	>500

The broken line indicates the average concentration level of dihydrostreptomycin in the blood caused by normal dosage.

aeruginosa strain 2 and Staph. aureus strain 4. Sensitive to dihydrostreptomycin initially were E. coli strain 3, A. aerog. strains 1 and 3, Staph. aureus strains 2 and 3, and the Staph. albus strain. On receiving dihydrostreptomycin in broth, the sensitivities of all the strains rose above the average concentration level. In addition, the resistance of many strains then exceeded 500 $\mu\text{g/ml}$. In daily cultures also the previously mentioned phenomenon was observed, that after one administration of dihydrostreptomycin the resistance on the next occasion was $>500 \mu\text{g/ml}$. However, a step-like increase in resistance was more common.

Sensitivities of Bacteria to Aureomycin after Cultivation in Broth Containing Dihydrostreptomycin (Table 23)

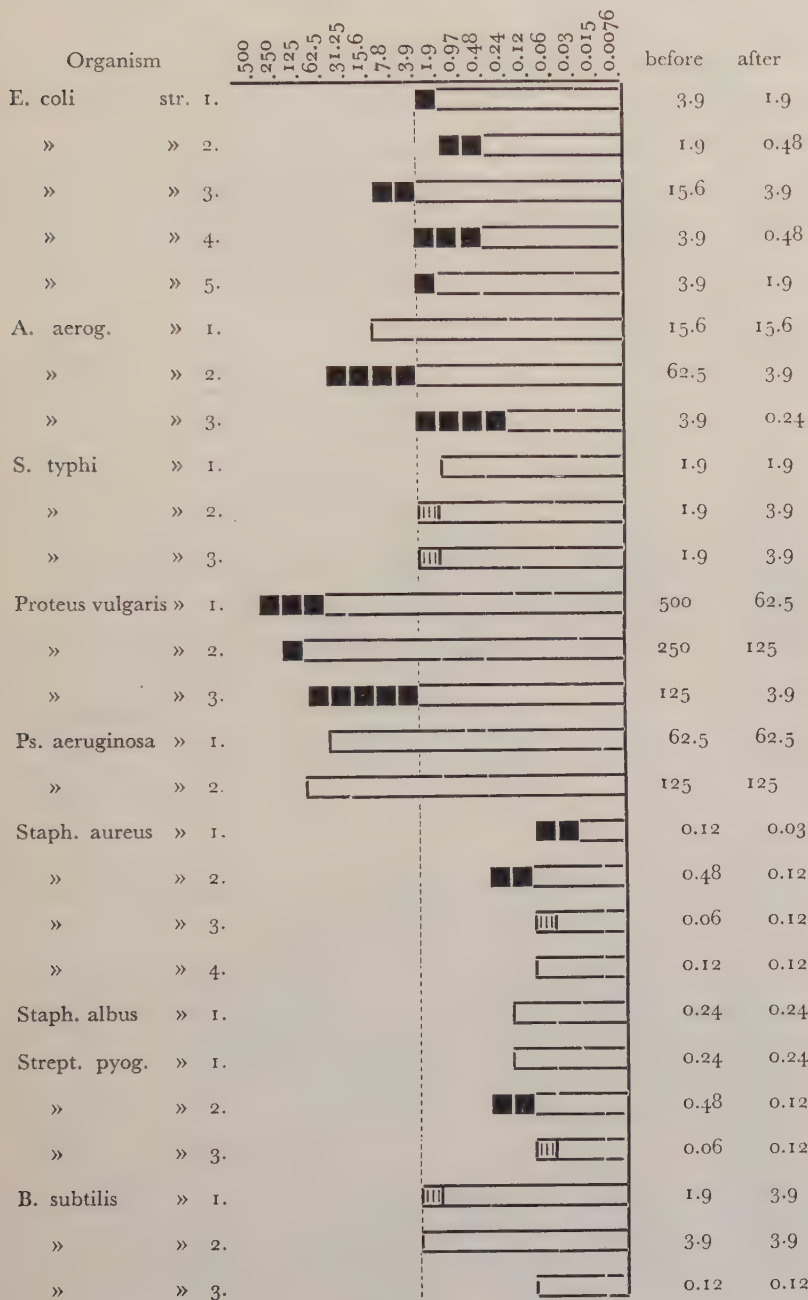
Alexander *et al.* (2) made several bacteria resistant to streptomycin and then studied their sensitivities to polymyxin B, chloromycetin and aureomycin. No strains were found among them resistant to these substances. Kaipainen (32) found that the sensitivity to aureomycin of three E. coli strains had increased slightly after cultivation in broth containing dihydrostreptomycin. Heilmann (30) found the same to have occurred with E. coli and A. aerog. strains. However, the increase in sensitivity was slight.

Table 23 shows that sensitivity to aureomycin increased slightly with some strains. Increased sensitivities to aureomycin with E. coli strain 4, A. aerog. strains 2 and 3, and Proteus vulgaris strains 1 and 3 were fairly distinct. The initial sensitivity of the last-mentioned strain was 125 $\mu\text{g/ml}$, and it increased from that to 3.9 $\mu\text{g/ml}$, or to the average concentration level. S. typhi strains did not gain in sensitivity, nor did the aureomycin-resistant Ps. aeruginosa strains become any more sensitive. A slight increase in sensitivity was also found with some staphylococcus and streptococcus strains.

The general impression remains that increased resistance to dihydrostreptomycin was not accompanied by increased resistance to aureomycin; sensitivities to aureomycin, in part, remained constant, *in part there were indications of increased sensitivity, and in a few cases the increase in resistance to aureomycin was quite distinct.* The shifts, therefore, were on the same lines as those occurring in sensitivities to dihydrostreptomycin when bacteria were cultivated in broth containing aureomycin.

TABLE 23

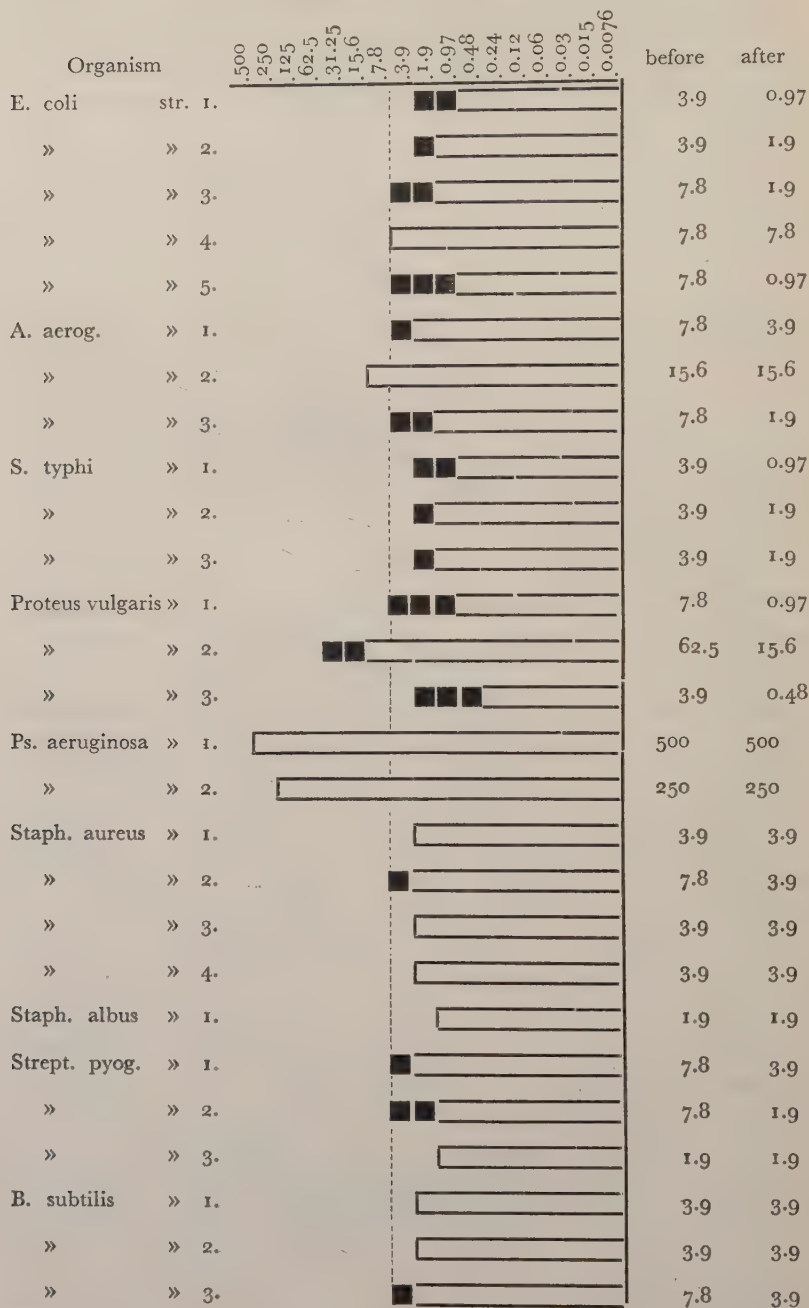
THE SENSITIVITY OF DIFFERENT BACTERIA TO AUREOMYCIN AFTER SEVEN TRANSFERS IN BROTH CONTAINING DIHYDROSTREPTOMYCIN.
(MIN. INHIB. CONC. $\mu\text{g/ml.}$)



The broken line indicates the average concentration level of aureomycin in the blood caused by normal dosage.

TABLE 24

THE SENSITIVITY OF DIFFERENT BACTERIA TO CHLOROMYCETIN AFTER SEVEN TRANSFERS IN BROTH CONTAINING DIHYDROSTREPTOMYCIN.
(MIN. INHIB. CONC. $\mu\text{g/ml.}$)



The broken line indicates the average concentration level of chloromycetin in the blood caused by normal dosage.

Sensitivities of Bacteria to Chloromycetin after Cultivation in Broth Containing Dihydrostreptomycin (Table 24)

Alexander *et al.* (2) found in their investigations that strains with increased resistance to streptomycin were not resistant to chloromycetin. Kaipainen's (32) investigations revealed that the resistance to chloromycetin of two *E. coli* strains decreased, while their resistance to dihydrostreptomycin increased, after cultivation in broth containing dihydrostreptomycin. In Heilman's (30) investigations the increased resistance of *E. coli* to streptomycin was accompanied by an increase in sensitivity to chloromycetin from 12.5 $\mu\text{g/ml}$ $\rightarrow$ 6.25 $\mu\text{g/ml}$. Accordingly, the sensitivity of his *A. aerog.* strain increased from 3.12 $\mu\text{g/ml}$ $\rightarrow$ 1.56 $\mu\text{g/ml}$. The shifts in sensitivity to chloromycetin were slight, and of the same range as those seen in Kaipainen's results with *E. coli* strains.

From Table 24 it can be seen that the cultivation of bacteria in broth containing dihydrostreptomycin resulted in increased sensitivities to chloromycetin with some strains. The increases in sensitivity to chloromycetin were on the same lines as to aureomycin (Table 23).

Although the increases in sensitivity were slight, it was striking that the final sensitivities seemed to fall off, with several strains, below the average concentration level.

Cultivation of bacteria in broth containing dihydrostreptomycin, hence, seemed to result in unchanged or slightly increased sensitivity to chloromycetin. The increase in sensitivity was not great, but distinct.

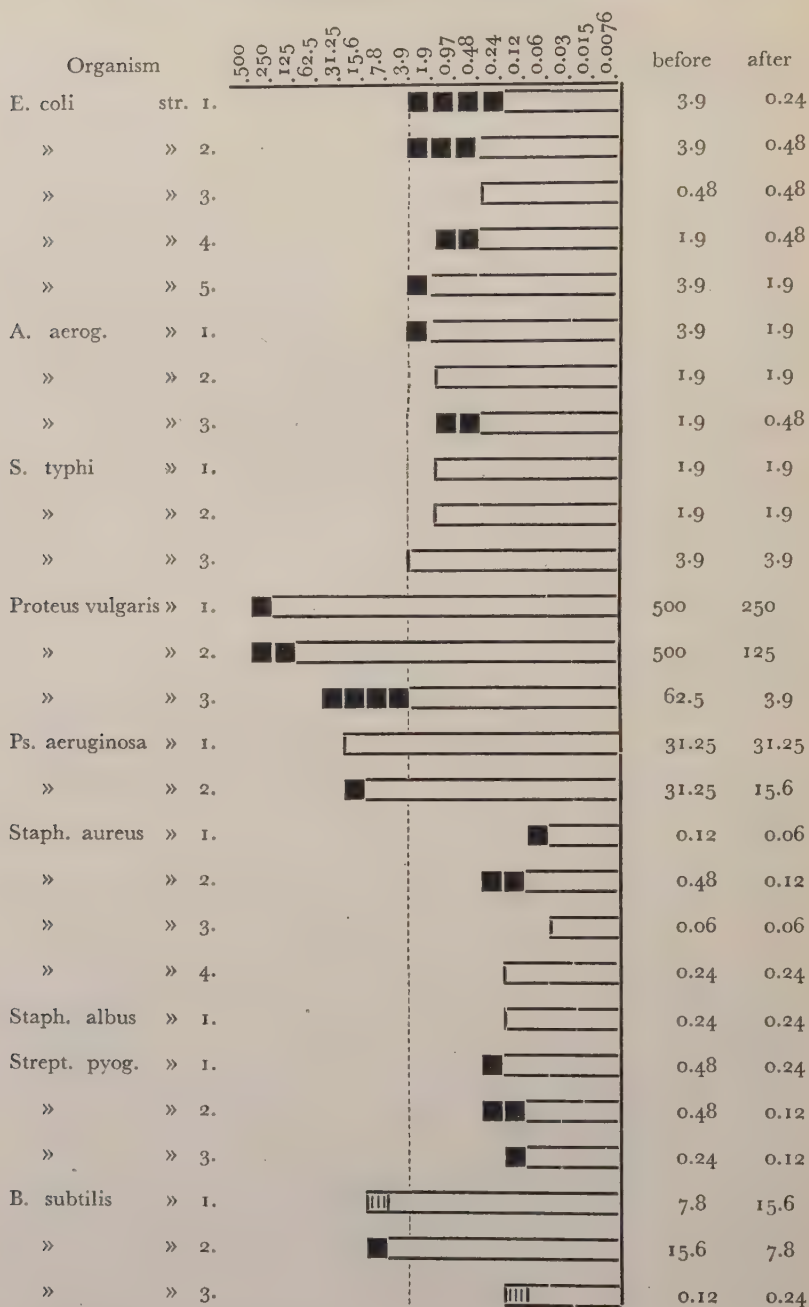
Sensitivities of Bacteria to Terramycin after Cultivation in Broth Containing Dihydrostreptomycin (Table 25)

In Heilman's (30) investigations increased resistance to streptomycin was accompanied, with one *E. coli* strain, by lowered resistance to terramycin from 3.12 $\mu\text{g/ml}$ $\rightarrow$ 1.56 $\mu\text{g/ml}$, whereas the sensitivity to terramycin of the *A. aerog.* strain remained unchanged. Even the change in sensitivity of the *E. coli* strain, admittedly, was slight.

Table 25 shows that cultivation in broth containing dihydrostreptomycin induced similar changes in the sensitivities of bacteria to terramycin as to aureomycin and chloromycetin. The terramycin

TABLE 25

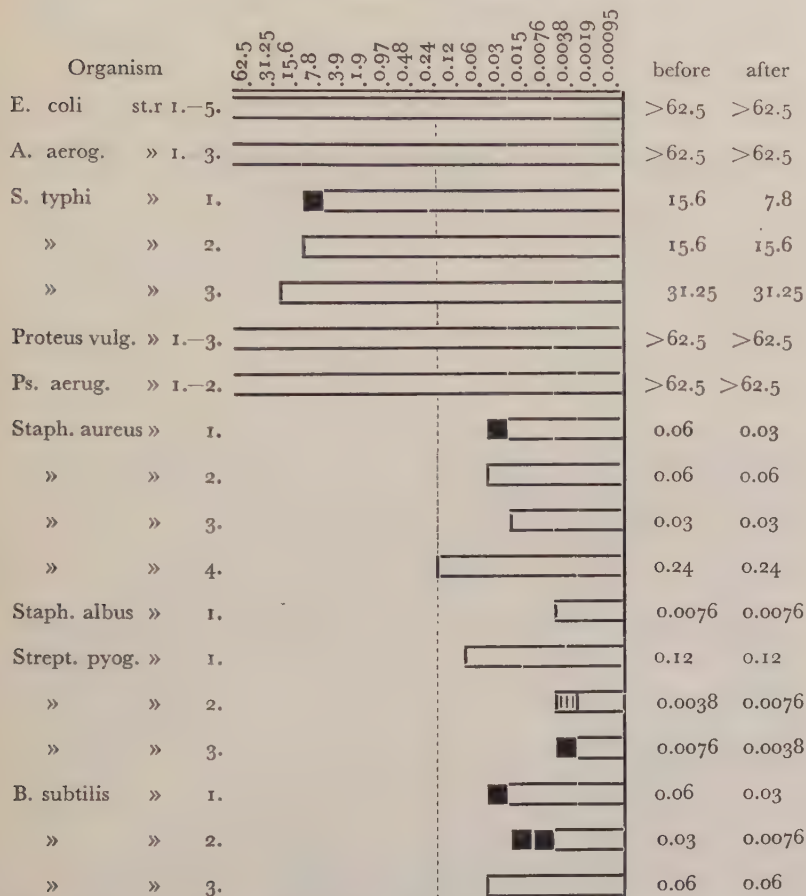
THE SENSITIVITY OF DIFFERENT BACTERIA TO TERRAMYCIN AFTER SEVEN TRANSFERS IN BROTH CONTAINING DIHYDROSTREPTOMYCIN.
(MIN. INHIB. CONC. $\mu\text{g/ml.}$)



The broken line indicates the average concentration level of terramycin in the blood caused by normal dosage.

TABLE 26

THE SENSITIVITY OF DIFFERENT BACTERIA TO PENICILLIN AFTER SEVEN TRANSFERS IN BROTH CONTAINING DIHYDROSTREPTOMYCIN.
(MIN. INHIB. CONC. units/ml.)



The broken line indicates the average concentration level of penicillin in the blood caused by normal dosage.

sensitivities of *E. coli* strains seemed to increase, most distinctly so with *E. coli* strain 1. The shifts in the sensitivity of *A. aerog.* strains were on the same lines as with *E. coli*, whereas the sensitivity to terramycin of *S. typhi* strains remained constant. *Proteus vulgaris* Strain 3 showed a considerable increase in sensitivity, its final sensitivity being that of the average concentration level.

The staphylococcus and streptococcus strains, initially sensitive to terramycin, revealed in part signs of increased sensitivity.

Cultivation of bacteria in broth containing dihydrostreptomycin seemed to result in unchanged or increased sensitivity to terramycin. The results were on the same lines as with aureomycin and chloromycetin (Tables 23 and 24).

Sensitivities of Bacteria to Penicillin after Cultivation in Broth Containing Dihydrostreptomycin (Table 26)

Cultivation of bacteria in broth containing aureomycin, chloromycetin or terramycin resulted in no significant changes in bacterial sensitivities to penicillin. Equally slight were the shifts in sensitivity to penicillin resulting from cultivation in broth containing dihydrostreptomycin (Table 26).

The cultivation of bacteria in broth containing dihydrostreptomycin, hence, did not appear to induce any changes in the sensitivities to penicillin.

Summary

Cultivation of bacteria in broth containing dihydrostreptomycin resulted in the following changes in the sensitivities of bacteria to the other antibiotics:

1. Sensitivities to aureomycin, chloromycetin and terramycin remained constant or increased slightly.
2. Sensitivities to penicillin remained constant.

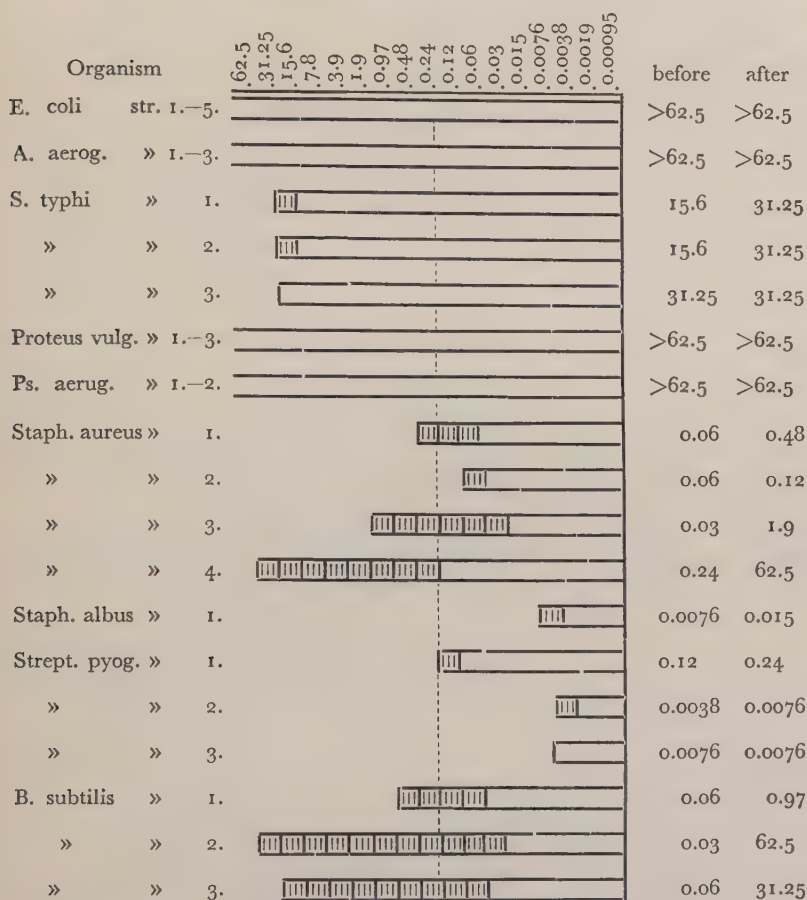
5. CULTIVATION IN BROTH CONTAINING PENICILLIN

Sensitivities of Bacteria to Penicillin after Cultivation in Broth Containing Penicillin (Table 27)

In earlier investigations into the increase of bacterial resistance in vitro, considerably heightened resistance has been induced by subcultures in nutrient media containing penicillin. Almost half the number of the bacterial strains employed in the present investigations were resistant to penicillin, for which reason they were cultivated

TABLE 27

THE SENSITIVITY OF DIFFERENT BACTERIA TO PENICILLIN AFTER SEVEN TRANSFERS IN BROTH CONTAINING PENICILLIN.
(MIN. INHIB. CONC. units/ml.)



The broken line indicates the average concentration level of penicillin in the blood caused by normal dosage.

by daily subcultures in broth with a penicillin concentration of 62.5 units/ml, while strains sensitive to penicillin were cultivated at the same time in rising concentrations of penicillin. Although in the present investigation seven transfers only were effected in broth containing penicillin, the sensitivity of the bacteria sensitive to

penicillin declined considerably in some cases. The increased resistance to penicillin of *Staphylococcus aureus* strains 1, 3 and 4 was distinct. The increase in resistance of *B. subtilis* strains was also considerable.

Sensitivities of Bacteria to Aureomycin after Cultivation in Broth Containing Penicillin (Table 28)

Although the *E. coli*, *A. aerog.*, *Proteus vulgaris* and *Ps. aeruginosa* strains were resistant (>62.5 units/ml) to penicillin, the possibility of cultivation in broth containing penicillin inducing some change in their sensitivities to aureomycin, chloromycetin, terramycin or dihydrostreptomycin was not entirely excluded. The results in Table 28, with regard to aureomycin, show that sensitivities to aureomycin, generally speaking, remained unchanged. The resistance to aureomycin of the *S. typhi* strains increased slightly, as did that of *E. coli* strains 2 and 4. Sensitivity to aureomycin of *Staphylococcus aureus* strains 1 and 2 and *B. subtilis* strain 1 was slightly increased.

Sensitivities of Bacteria to Chloromycetin after Cultivation in Broth Containing Penicillin (Table 29)

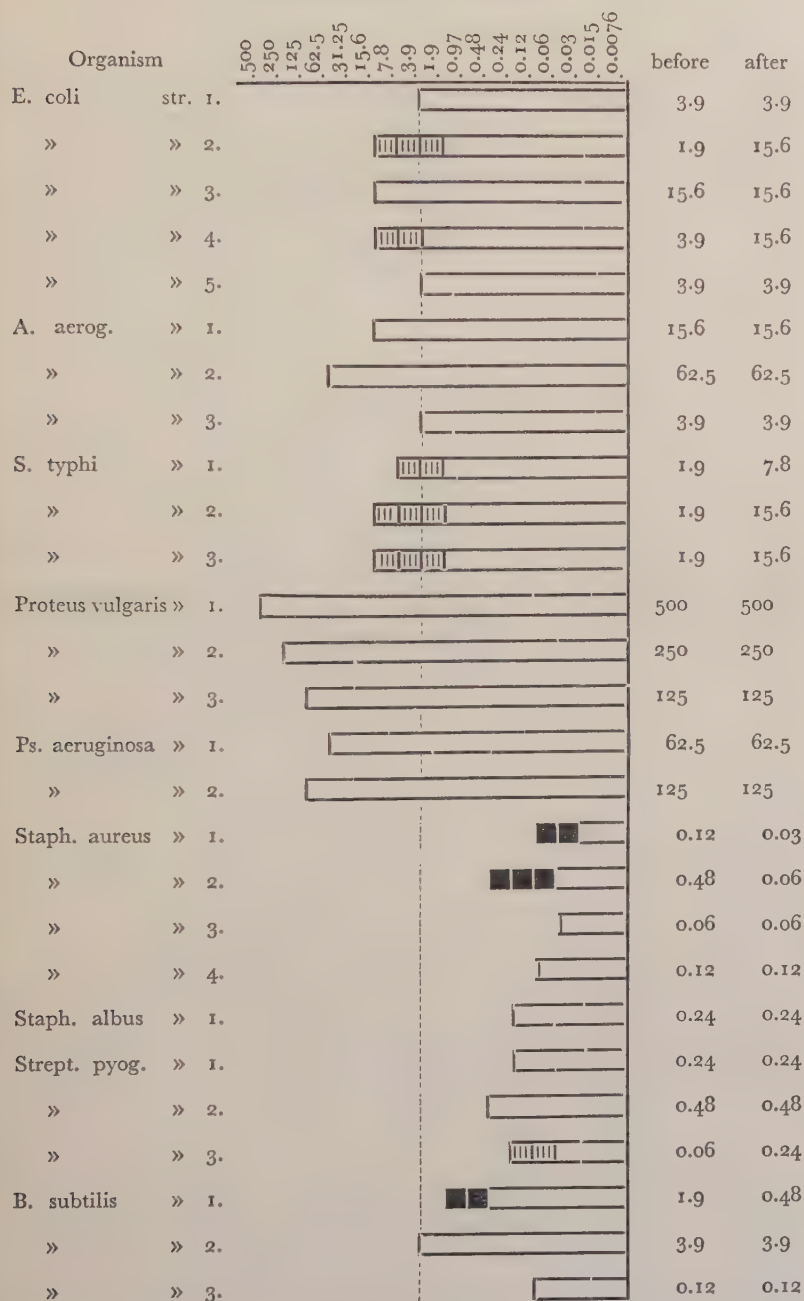
The changes in the sensitivities of bacteria to chloromycetin (Table 29) resulting from subcultures in broth containing penicillin were approximately similar to the changes with regard to aureomycin (Table 28).

Sensitivities of Bacteria to Terramycin after Cultivation in Broth Containing Penicillin (Table 30)

Table 30 shows that the sensitivities to terramycin also remained almost unchanged. However, it is remarkable that the sensitivity of the *S. typhi* strains to terramycin showed signs of decreasing. The same phenomenon was revealed by these strains with regard to aureomycin (Table 28). It can be said that the changes in sensitivities to terramycin resulting from the cultivation of bacteria in broth containing penicillin are comparable to the corresponding sensitivity changes to aureomycin and chloromycetin.

TABLE 28

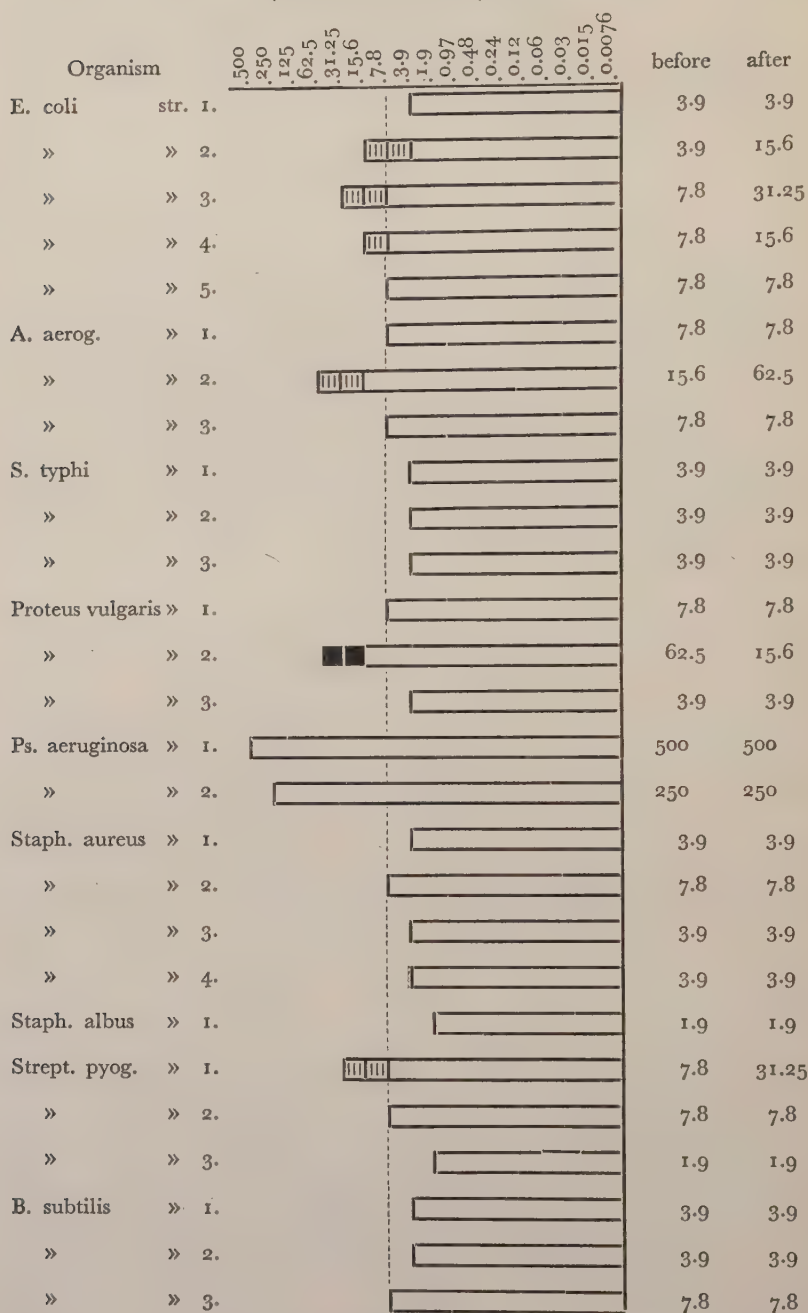
THE SENSITIVITY OF DIFFERENT BACTERIA TO AUREOMYCIN AFTER SEVEN TRANSFERS IN BROTH CONTAINING PENICILLIN.
(MIN. INHIB. CONC. $\mu\text{g/ml.}$)



The broken line indicates the average concentration level of aureomycin in the blood caused by normal dosage.

TABLE 29

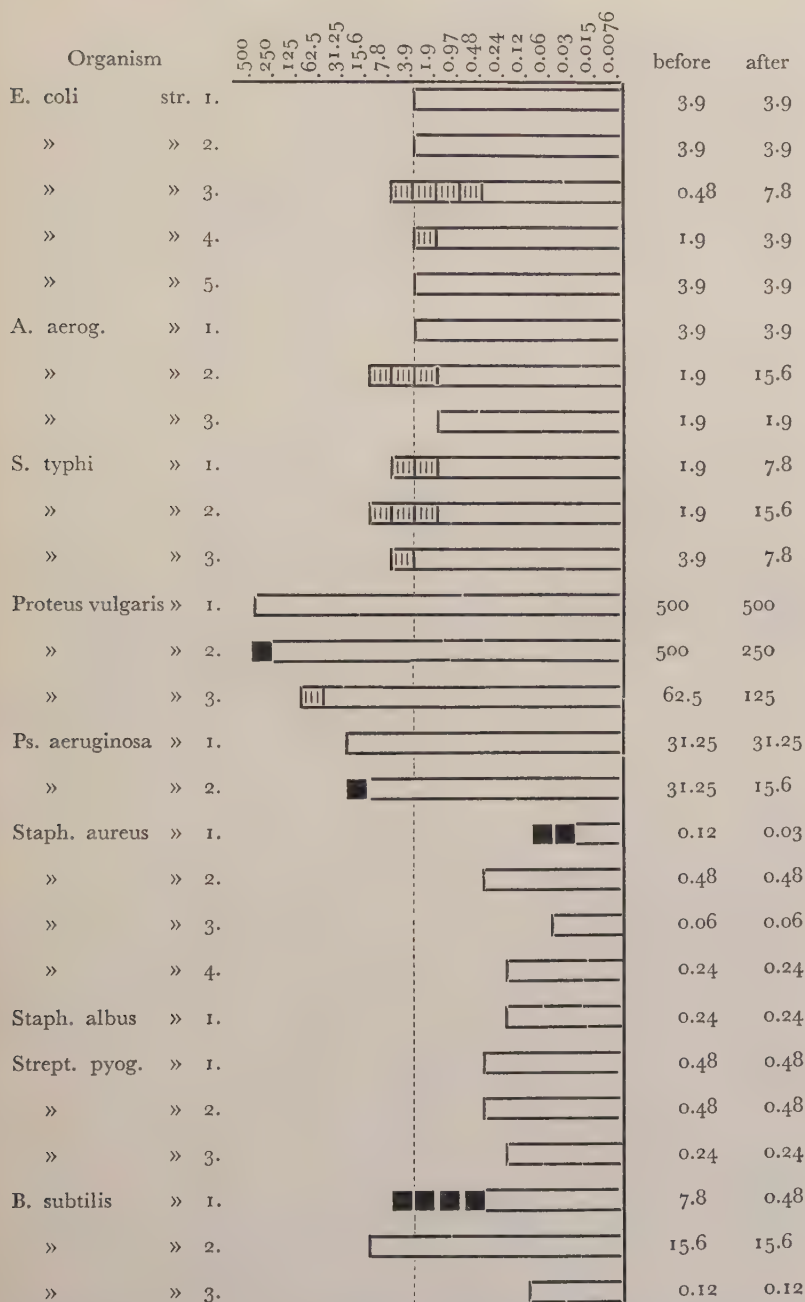
THE SENSITIVITY OF DIFFERENT BACTERIA TO CHLOROMYCETIN AFTER SEVEN TRANSFERS IN BROTH CONTAINING PENICILLIN.
(MIN. INHIB. CONC. $\mu\text{g/ml.}$)



The broken line indicates the average concentration level of chloromycetin in the blood caused by normal dosage.

TABLE 30

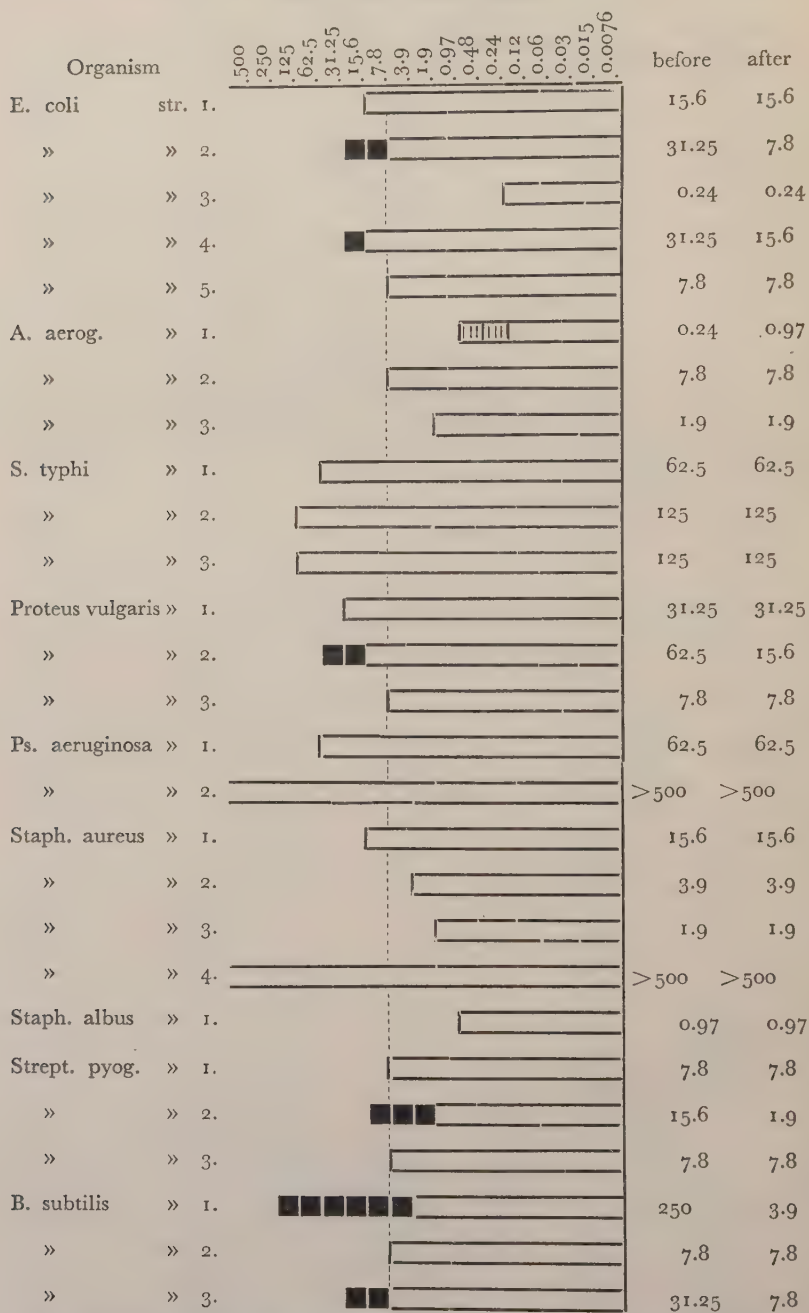
THE SENSITIVITY OF DIFFERENT BACTERIA TO TERRAMYCIN AFTER SEVEN TRANSFERS IN BROTH CONTAINING PENICILLIN.
(MIN. INHIB. CONC. $\mu\text{g/ml.}$)



The broken line indicates the average concentration level of terramycin in the blood caused by normal dosage.

TABLE 31

THE SENSITIVITY OF DIFFERENT BACTERIA TO DIHYDROSTREPTOMYCIN AFTER SEVEN TRANSFERS IN BROTH CONTAINING PENICILLIN.
(MIN. INHIB. CONC. $\mu\text{g/ml.}$)



The broken line indicates the average concentration level of dihydrostreptomycin in the blood caused by normal dosage.

Sensitivities of Bacteria to Dihydrostreptomycin after Cultivation in Broth Containing Penicillin (Table 31)

Graessle and Frost (24) cultivated *Staphylococcus aureus* strains in nutrient medium containing penicillin and observed at the same time the shifts occurring in the sensitivities to streptomycin with resistance to penicillin increasing. They found that the resistance to streptomycin of two strains increased simultaneously with the resistance to penicillin. This occurred with the *Staph. aureus* strains, which showed the greatest increase in resistance to penicillin.

The changes seen in Table 31 in the sensitivities of bacteria to dihydrostreptomycin are slight and are comparable with the results in Tables 28—30. However, the increase in sensitivity to dihydrostreptomycin of *B. subtilis* strain 1 was considerable.

On the other hand, the sensitivity to dihydrostreptomycin of *Staph. aureus* strains did not undergo any change although, bearing in mind Graessle's and Frost's results, something of the sort could have been expected.

Hence, the changes occurring in the bacterial sensitivities to dihydrostreptomycin as a result of cultivation in broth containing penicillin, were slight, and corresponded to the changes that took place with regard to aureomycin, chloromycetin and terramycin.

Summary

Cultivation of bacteria in broth containing penicillin did not result in any distinct changes in the sensitivities to aureomycin, chloromycetin, terramycin or dihydrostreptomycin, apart from certain occasional exceptions.

6. DISCUSSION

In the present investigation various bacterial species were cultivated in broth containing a certain antibiotic, and the resulting changes in their sensitivities to the other antibiotics were studied. The results were on the same lines as those attained in earlier experiments by Kaipainen (32) and Heilmann (30). The observations, in their main features, were as follows:

1. Increased resistance to aureomycin was accompanied by increased resistance to chloromycetin and terramycin, unchanged or increased sensitivity to dihydrostreptomycin, and no change in sensitivity to penicillin.
2. Increased resistance to chloromycetin was accompanied by increased resistance to aureomycin and terramycin, unchanged or increased sensitivity to dihydrostreptomycin, and no change in sensitivity to penicillin.
3. Increased resistance to terramycin was accompanied by increased resistance to aureomycin and chloromycetin, unchanged or slightly increased sensitivity to dihydrostreptomycin, and no change in sensitivity to penicillin.
4. Increased resistance to dihydrostreptomycin was accompanied by unchanged or slightly increased sensitivity to aureomycin, chloromycetin and terramycin, and no change in sensitivity to penicillin.
5. Cultivation of bacteria in broth containing penicillin did not result in any general changes in sensitivity to aureomycin, chloromycetin, terramycin or dihydrostreptomycin.
6. Changes listed under Items 1—4 occurred generally with the Gram-negative rods, *E. coli*, *A. aerog.* and *S. typhi* strains, employed. Such changes also took place with the *Proteus vulgaris* and *Ps. aeruginosa* strains, but were somewhat less marked. With *Staph. aureus* and *albus*, *Strept. pyogenes* and *B. subtilis* strains the changes mentioned under Items 1—4, in general, did not occur. The above results also include exceptions.

The simultaneous increases in resistance revealed by cross testing, generally, were not very great, but in many cases (Table 32) the

TABLE 32

INCREASE IN RESISTANCE OF BACTERIA, RESULTING FROM THEIR CULTIVATION IN BROTH CONTAINING AUREOMYCIN, CHLOROMYCETIN OR TERRAMYCIN, TO THE OTHER TWO OF THESE ANTIBIOTICS.

Resistance increased four-fold or more

1. + with sensitivity at the same time exceeding the average therapeutically effective concentration level,
2. (+) with initial sensitivity exceeding the average concentration level,
3. [+] with induced sensitivity not exceeding the average concentration level.

Cultivation in broth containing		Aureomycin		Chloromycetin		Terramycin	
Increase in resistance to		Chlo- rom.	Terram.	Aureom.	Terram.	Aureom.	Chlo- rom.
E. coli	str. 1.			+			
»	» 2.	+	+	+	+	+	+
»	» 3.	+	+		[+]	(+)	+
»	» 4.	+	+	+	+	+	+
»	» 5.	+	+	+	+	+	+
A. aerog.	» 1.	+	+	(+)		(+)	+
»	» 2.	(+)	+		+		
»	» 3.	+	+	+		+	+
S. typhi	» 1.	+	+	+	+	+	+
»	» 2.	+	+	+	+	+	+
»	» 3.			+	+	+	+
Proteus vulgaris	» 1.	+					
»	» 2.			(+)		(+)	(+)
»	» 3.	+	(+)			(+)	
Ps. aeruginosa	» 1.		(+)	(+)	(+)	(+)	
»	» 2.	(+)	(+)				
Staph. aureus	» 1.						
»	» 2.						
»	» 3.		[+]				
»	» 4.						
Staph. albus	» 1.					[+]	
Strept. pyog.	» 1.						
»	» 2.						
»	» 3.					[+]	
B. subtilis	» 1.		(+)			+	
»	» 2.					+	
»	» 3.		[+]				

average therapeutically effective concentration level (aureomycin, chloromycetin, terramycin), however, was exceeded. If a similar simultaneous increase in resistance to these antibiotics were to take place *in vivo* also, it might spoil our possibilities of treating infections with all of these three antibiotics.

The staphylococci and streptococci, initially sensitive to aureomycin and penicillin, did not acquire simultaneous resistance to both these substances. For this reason the position with these bacteria might possibly be more favourable, as these antibiotics could be employed consecutively where no positive result is gained from treatment with the first one.

The simultaneous increases in sensitivities to aureomycin, chloromycetin, and terramycin accompanying increased resistance to dihydrostreptomycin were slight, but so much on the same lines that the phenomenon had to be considered a fact. The same is true of increases in sensitivities to dihydrostreptomycin after cultivation in broth containing aureomycin, chloromycetin or terramycin. Is it perhaps possible that this increased sensitivity to dihydrostreptomycin results *e. g.* from aureomycin eliminating the strains most resistant to dihydrostreptomycin, leaving only the strains sensitive to dihydrostreptomycin? Can it be assumed that this might already have taken place in the course of the first subculture in broth containing aureomycin?

Searching for an explanation of the changes in sensitivity mentioned under Items 1—4 theoretical speculation may be permitted. As, according to the theory of spontaneous mutation, the antibiotic, with increasing resistance, has no significance other than the elimination of susceptible individuals, the explanation based on this theory — that the resistance increases simultaneously — would seem natural and simple. For instance, when resistance to aureomycin increases, susceptible specimens would be eliminated through the action of aureomycin, and the resistant specimens would be left, which, as a result of spontaneous mutation, have become resistant to chloromycetin and terramycin as well.

At the same time it appeared that *aureomycin, chloromycetin and terramycin, in their mode of action, constituted a group somehow opposed to dihydrostreptomycin*. From this it could be expected that combinations such as

dihydrostreptomycin	+ aureomycin,
»	+ chloromycetin,
»	+ terramycin,

might be effective in preventing the resistance from increasing.

Without trying to explain the ultimate reason for the increase in bacterial resistance to the primary antibiotic, any more than for the resulting simultaneous shift in sensitivity to another antibiotic, the author has considered it necessary to carry out certain investigations reported on in the next chapter, closely related to the phenomena mentioned under Items 1-4 and dealing with the problems presented there.

B. COMPARATIVE EXPERIMENTS

1. COMPARATIVE EXPERIMENTS WITH A MORE SENSITIVE STRAIN ISOLATED FROM A LESS SENSITIVE A.AEROG. PARENT STRAIN

The sensitivities to antibiotics of the different individuals of any one bacterial strain may be different (8). The increase in resistance to primary antibiotics, and simultaneous shifts in sensitivity to other antibiotics, reported in the initial part of the present paper, can be presumed to have taken place with the most resistant individuals of the bacterial strain, as the bacteria employed in the experiments were always taken from tubes with the highest antibiotic concentration possible which did not prevent growth. For this reason, comparative experiments were carried out to ascertain whether, with a more sensitive strain isolated from a certain parent strain, cultivation in broth containing one antibiotic resulted in shifts in sensitivity to the other antibiotics also.

The test organism was *A. aerogenes* Strain 3 (a strain taken from routine samples). Employing an overnight broth culture, the strain was divided into two parts. From the first part, here called Strain A I, immediate sensitivity determinations were made, and the raising of resistance to primary antibiotics was continued, upon which the usual cross testing was effected. From the second half, a loopful was placed in 5 ml of broth, a loopful of which was then transferred to a Petri dish. This was allowed to stand overnight in an incubator. In the morning a part of a colony, using the tip of the loop, was

transferred into fresh broth, from which, again, a loopful was transferred onto a Petri dish. This was repeated for seven days, with the object of finding a specimen the sensitivity of which to a primary antibiotic would exceed that of A I. It proved that a strain, A II, had been separated, with a sensitivity to dihydrostreptomycin of $1.9 \mu\text{g/ml}$, whereas that of the parent strain was $62.5 \mu\text{g/ml}$. Simultaneously, a part of the parent strain was cultivated by daily subcultures in pure broth and another part on a Petri dish. Their sensitivities were found to have remained unchanged.

A review of the results in Table 33 shows that increases in resistance to primary antibiotics were identical with both these strains, apart from the final dihydrostreptomycin values, in which the initial values were $62.5 \mu\text{g/ml}$ and $1.9 \mu\text{g/ml}$. The results of the cross testing revealed very little difference with regard to sensitivities to aureo-

TABLE 33

THE SENSITIVITIES OF A. AEROG. STR. A I AND A II TO VARIOUS ANTIBIOTICS AFTER CULTIVATION IN BROTH CONTAINING AUREOMYCIN, CHLOROMYCETIN, TERRAMYCIN, OR DIHYDROSTREPTOMYCIN.

A. aerog.	Cultiv. in broth containing	Sensitivity ($\mu\text{g/ml}$)				
			Aureo- mycin	Chloro- mycetin	Terra- mycin	Dihydro- strepto- mycin
Str. A I	Aureo- mycin	before	3.9	7.8	1.9	62.5
		after	125	125	62.5	7.8
» A II	»	before	3.9	7.8	1.9	1.9
		after	125	125	31.25	0.24
» A I	Chloro- mycetin	before	3.9	7.8	1.9	62.5
		after	31.25	500	3.9	0.97
» A II	»	before	3.9	7.8	1.9	1.9
		after	15.6	500	1.9	0.12
» A I	Terra- mycin	before	3.9	7.8	1.9	62.5
		after	62.5	62.5	31.25	1.9
» A II	»	before	3.9	7.8	1.9	1.9
		after	125	250	31.25	0.24
» A I	Dihydro- strept.	before	3.9	7.8	1.9	62.5
		after	0.24	1.9	0.48	>500
» A II	»	before	3.9	7.8	1.9	1.9
		after	0.24	1.9	0.48	250

mycin, chloromycetin and terramycin. In the final sensitivities to dihydrostreptomycin a difference of approximately the same extent persisted between Strains A I and A II. These differences reflect the different initial sensitivities to dihydrostreptomycin.

The result of this experiment can be said to be that the simultaneous changes in sensitivity to other antibiotics accompanying increased resistance to one antibiotic were on the same lines with Strains A I and A II. Of these, A I represented a strain possibly containing numerous individuals of varying sensitivity to antibiotics, while the latter, A II, represented a selected strain isolated from the former, more sensitive to dihydrostreptomycin.

2. ON THE DURATION OF THE CHANGED SENSITIVITIES DETECTED IN CROSS TESTING

Duration of sensitivities in daily broth cultures. After a cross testing had been effected from *A. aerogenes* Strain 3, mutations made resistant to each primary antibiotic were cultivated by daily subcultures in broth for four weeks; in the subsequent experiments it was found that the increased resistance to primary antibiotics had remained constant. The cross testing now effected showed that the sensitivities to the other antibiotics were still the same in extent as in the first cross testing.

The changes in sensitivity ascertained by cross testing cannot be considered temporary phenomena with this *A. aerogenes* strain. Particularly noteworthy is the fact that the increases in sensitivities were permanent, and could not be due *e. g.* to poor growth conditions spoiled by antibiotics.

Duration of increased sensitivity, in broth culture in the presence of an antibiotic. In the previous chapter it was found that the changed sensitivities detected by cross testing were of a permanent character in daily subcultures in antibiotic-free broth. The following experiments studied the reduction in the increased sensitivities of *E. coli* Strain 5 detected by cross testing; the *E. coli* strain, sensitised to a certain antibiotic, was cultivated in broth containing that antibiotic.

By cultivating *E. coli* Strain 5 in broth containing dihydrostreptomycin its aureomycin-sensitivity was increased. This strain was subsequently cultivated in broth with rising aureomycin concentrations, and the increase in resistance was compared with the

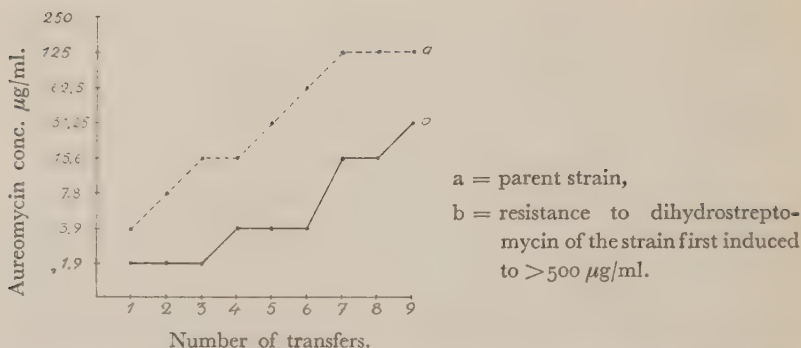


Fig. 2. Increase in the resistance to aureomycin of *E. coli* strain 5 cultivated by daily transfers in broth containing aureomycin.

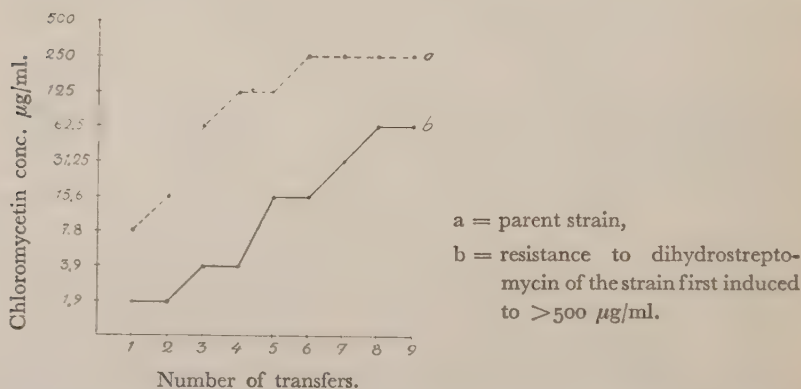


Fig. 3. Increase in the resistance to chloromycetin of *E. coli* strain 5 cultivated by daily transfers in broth containing chloromycetin.

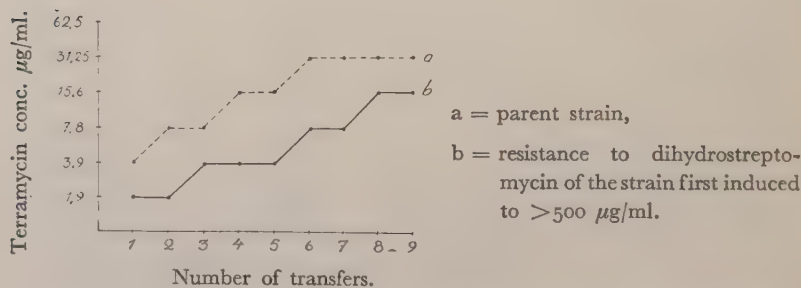


Fig. 4. Increase in the resistance to terramycin of *E. coli* strain 5 cultivated by daily transfers in broth containing terramycin.

increase in resistance to aureomycin of the parent strain. Corresponding experiments were carried out with chloromycetin and terramycin. A review of the results, in Figs. 2, 3 and 4, shows that the resistance of the parent strain and the strain sensitised by the action of dihydrostreptomycin increased parallelly. The differences in sensitivity present in the initial values are still discernible at the end of the curves.

The increased sensitivity to dihydrostreptomycin of *E. coli* strain 5 acquired from cultivation in broth containing aureomycin, chloromycetin or terramycin showed a similar reduction in corresponding experiments.

3. COMPARATIVE EXPERIMENTS WITH DIFFERENT NUTRIENT MEDIA

The experiments to study the shifts in sensitivities induced by antibiotics were carried out with broth as the nutrient medium. Corresponding experiments were effected, also, with other nutrient media, viz. peptone water, tryptose-phosphate, and Kirchner medium.

<i>Broth</i>		<i>Peptone Water</i>	
NaCl	5.0	NaCl	5.0
Peptone (Witte)	10.0	Peptone (Witte)	10.0
Bouillon	1000.0	Aq.dest.	1000.0
<i>Tryptose-Phosphate</i>		<i>Kirchner Modification</i>	
Bacto-Tryptose (Difco) ...	20.0	$\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$	3.0
Yeast extr. (Difco)	5.0	KH_2PO_4	4.0
NaCl	8.0	$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.6
Glucose	0.5	Sodium Citrate $\cdot 2\text{H}_2\text{O}$	2.5
Phosphate buff. (pH 7.6) ..	22.0	Asparagine	5.0
(KH_2PO_4	1.45	Glycerin	20.0
$\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$...	7.60	Aq. dest.	1000.0
Aq.dest. ad	100.0)	Sterilised, then bovine serum added	100.0
Aq. dest.	1000.0		

The test organism employed was *E. coli* Strain 5. In sensitivity determinations and when inducing resistance in the bacterial strain, the same method was employed as in the previous experiments. The sensitivities to the primary antibiotics varied somewhat with different nutrient media. The sensitivities in broth, peptone water and tryptose-phosphate corresponded most closely to one another. In Kirchner medium the sensitivities to aureomycin, chloromycetin and terramycin were higher than in broth, peptone water and tryptose-phosphate. The sensitivity to penicillin of this *E. coli* strain was

TABLE 34

INCREASE IN RESISTANCE TO AUREOMYCIN OF *E. COLI* STRAIN 5 IN THE COURSE OF SEVEN TRANSFERS IN DIFFERENT NUTRIENT MEDIA CONTAINING AUREOMYCIN IN RISING QUANTITIES.
(MINIM. INHIB. CONC. $\mu\text{g/ml.}$)

Nutrient medium	Number of transfers							Increase
	1	2	3	4	5	6	7	
Broth	3.9	7.8	15.6	15.6	31.25	62.5	125	32-fold
Peptone water	1.9	3.9	15.6	15.6	15.6	31.25	62.5	32 »
Tryptose-phosphate	0.97	3.9	7.8	15.6	15.6	15.6	31.25	32 »
Kirchner medium	0.12	0.24	0.48	0.97	0.97	1.9	1.9	16 »

TABLE 35

INCREASE IN RESISTANCE TO CHLOROMYCETIN OF *E. COLI* STRAIN 5 IN THE COURSE OF SEVEN TRANSFERS IN DIFFERENT NUTRIENT MEDIA CONTAINING CHLOROMYCETIN IN RISING QUANTITIES.
(MINIM. INHIB. CONC. $\mu\text{g/ml.}$)

Nutrient medium	Number of transfers							Increase
	1	2	3	4	5	6	7	
Broth	7.8	15.6	62.5	125	125	250	250	32-fold
Peptone water	7.8	15.6	31.25	62.5	125	125	125	16 »
Tryptose-phosphate	7.8	15.6	62.5	125	125	125	250	32 »
Kirchner medium	1.9	3.9	3.9	3.9	7.8	15.6	15.6	8 »

TABLE 36

INCREASE IN RESISTANCE TO TERRAMYCIN OF E. COLI STRAIN 5 IN THE COURSE OF SEVEN TRANSFERS IN DIFFERENT NUTRIENT MEDIA CONTAINING TERRAMYCIN IN RISING QUANTITIES.
(MINIM. INHIB. CONC. $\mu\text{g/ml.}$)

Nutrient medium	Number of transfers							Increase
	1	2	3	4	5	6	7	
Broth	3.9	7.8	7.8	15.6	15.6	31.25	31.25	8-fold
Peptone water	3.9	3.9	7.8	15.6	31.25	31.25	31.25	8 »
Tryptose-phosphate	3.9	7.8	7.8	7.8	7.8	7.8	15.6	4 »
Kirchner medium	0.24	0.24	0.24	0.48	0.48	0.48	0.48	2 »

TABLE 37

INCREASE IN RESISTANCE TO DIHYDROSTREPTOMYCIN OF E. COLI STRAIN 5 IN THE COURSE OF SEVEN TRANSFERS IN DIFFERENT NUTRIENT MEDIA CONTAINING DIHYDROSTREPTOMYCIN IN RISING QUANTITIES.
(MINIM. INHIB. CONC. $\mu\text{g/ml.}$)

Nutrient medium	Number of transfers							Increase
	1	2	3	4	5	6	7	
Broth	7.8	31.25	31.25	62.5	250	500	>500	>128-fold
Peptone water ...	7.8	7.8	15.6	62.5	500	>500	>500	>128 »
Tryptose-phosphate	7.8	31.25	125	500	>500	>500	>500	>128 »
Kirchner medium	7.8	15.6	15.6	31.25	62.5	62.5	125	16 »

>62.5 units/ml in all media. Efforts to raise the resistance to the primary antibiotics revealed that, generally, it increased a little more slowly, and least in Kirchner medium. The difference was most distinct in the increase in resistance to dihydrostreptomycin (Tables 34, 35, 36, 37).

Tables 38, 39, 40, 41 show that the results from cross testing were parallel with different nutrient media. The range of the shift in sensitivity varied slightly, but no contradictions were present. The

most considerable deviation can be seen in Table 40, where it is clear that acquired resistance to terramycin was accompanied by a 16-fold increase in resistance to chloromycetin, whereas in tryptose-phosphate the sensitivity to chloromycetin showed no change. Nor did G o c k e *et al.* (23) find, in their experiments on agar plates,

TABLE 38

SENSITIVITIES OF *E. COLI* STRAIN 5 TO AUREOMYCIN, CHLOROMYCETIN, TERRAMYCIN AND DIHYDROSTREPTOMYCIN AFTER SEVEN TRANSFERS IN DIFFERENT NUTRIENT MEDIA CONTAINING AUREOMYCIN.
(MIN. INHIB. CONC. $\mu\text{g/ml.}$)

Nutrient medium	Aureomycin		Chloromycetin		Terramycin		Dihydrostreptomycin	
	before	after	before	after	before	after	before	after
Broth	3.9	125	7.8	125	3.9	62.5	7.8	3.9
Peptone water ...	1.9	62.5	7.8	62.5	3.9	31.25	7.8	1.9
Tryptose-phosphate *	0.97	31.25	7.8	31.25	3.9	31.25	7.8	1.9
Kirchner medium	0.06	1.9	1.9	7.8	0.24	0.97	7.8	3.9

TABLE 39

THE SENSITIVITIES OF *E. COLI* STRAIN 5 TO CHLOROMYCETIN, AUREOMYCIN, TERRAMYCIN AND DIHYDROSTREPTOMYCIN AFTER SEVEN TRANSFERS IN DIFFERENT NUTRIENT MEDIA CONTAINING CHLOROMYCETIN.
(MINIM. INHIB. CONC. $\mu\text{g/ml.}$)

Nutrient medium	Chloromycetin		Aureomycin		Terramycin		Dihydrostreptomycin	
	before	after	before	after	before	after	before	after
Broth	7.8	250	3.9	31.25	3.9	15.6	7.8	3.9
Peptone water ...	7.8	125	1.9	15.6	3.9	31.25	7.8	1.9
Tryptose-phosphate	7.8	250	0.97	31.25	3.9	15.6	7.8	1.9
Kirchner medium	1.9	15.6	0.06	0.24	0.24	0.48	7.8	3.9

* In a control series in which this strain was cultivated in tryptose-phosphate without aureomycin, the aureomycin resistance in the course of 7 transfers increased from 0.97 μg to 3.9 μg . At the same time, however, the sensitivity of this *E. coli* strain to any of the antibiotics showed no change when cultivated in broth only, Kirchner medium or peptone water.

TABLE 40

SENSITIVITIES OF *E. COLI* STRAIN 5 TO TERRAMYCIN, AUREOMYCIN, CHLOROMYCETIN AND DIHYDROSTREPTOMYCIN AFTER SEVEN TRANSFERS IN DIFFERENT NUTRIENT MEDIA CONTAINING TERRAMYCIN.
(MINIM. INHIB. CONC. $\mu\text{g/ml}$)

Nutrient medium	Terramycin		Aureomycin		Chloromycetin		Dihydrostreptomycin	
	before	after	before	after	before	after	before	after
Broth	3.9	31.25	3.9	125	7.8	125	7.8	1.9
Peptone water ...	3.9	31.25	1.9	31.25	7.8	62.5	7.8	1.9
Tryptose-phosphate	3.9	15.6	0.97	1.9	7.8	7.8	7.8	1.9
Kirchner medium	0.24	0.48	0.06	0.48	1.9	15.6	7.8	3.9

TABLE 41

SENSITIVITIES OF *E. COLI* STRAIN 5 TO DIHYDROSTREPTOMYCIN, AUREOMYCIN, CHLOROMYCETIN AND TERRAMYCIN AFTER SEVEN TRANSFERS IN DIFFERENT NUTRIENT MEDIA CONTAINING DIHYDROSTREPTOMYCIN.
(MINIM. INHIB. CONC. $\mu\text{g/ml}$)

Nutrient medium	Dihydrostreptomycin		Aureomycin		Chloromycetin		Terramycin	
	before	after	before	after	before	after	before	after
Broth	7.8	>500	3.9	1.9	7.8	0.97	3.9	1.9
Peptone water ...	7.8	>500	1.9	0.48	7.8	1.9	3.9	1.9
Tryptose-phosphate	7.8	>500	0.97	0.24	7.8	0.97	3.9	0.97
Kirchner medium	7.8	125	0.06	0.015	1.9	0.48	0.24	0.12

that the increased resistance to terramycin was accompanied by increased resistance to chloromycetin.

The control series, in which *E. coli* was cultivated by daily subcultures in antibiotic-free media, revealed that the sensitivity to aureomycin of the *E. coli* strain cultivated in tryptose-phosphate decreased from 0.97 $\mu\text{g/ml}$ to 3.9 $\mu\text{g/ml}$.

The sensitivity to penicillin, in cross testing, of *E. coli*, in all nutrient media, was >62.5 units/ml, and cultivation in broth, peptone water, tryptose-phosphate or Kirchner medium containing penicillin did not result in shifts in the sensitivities of *E. coli* to the other antibiotics.

4. THE EFFECT OF DIFFERENT ANTIBIOTIC CONCENTRATIONS AND OF VARYING CULTIVATION PERIODS ON THE SENSITIVITIES

Using *E. coli* strain 4 as the test organism, experiments were carried out to ascertain the extent to which the effect of different antibiotic concentrations and varying cultivation periods were capable of inducing increased resistance to any one antibiotic and simultaneous changes in sensitivities to the other antibiotics.

E. coli strain 4 was divided into four parts. The first part, E I, was kept for 24 hours in broth in a refrigerator in four different tubes, one for each antibiotic employed (1 $\mu\text{g/ml}$ aureomycin, chloromycetin, terramycin and dihydrostreptomycin). Subsequently, a loopful from each tube was transferred into fresh broth, which was allowed to stand overnight in an incubator (+37°C). The sensitivities to the different antibiotics of the strains in each tube were then determined. The purpose was to ascertain whether sensitivities change while the broth contains an antibiotic but growth is inhibited by the low temperature.

The second part, E II, was kept in broth with similar (1 $\mu\text{g/ml}$) antibiotic concentrations for 24 hours in an incubator, after which sensitivity determinations were carried out. The purpose was to ascertain whether cultivation for 24 hours in broth containing an antibiotic results in changes in sensitivity to other antibiotics.

The third part, E III, was cultivated for 7 days in broth with similar (1 $\mu\text{g/ml}$) antibiotic concentrations, after which sensitivities to the other antibiotics were determined. The purpose was to discover the effects of an extended cultivation period on the final results.

The fourth part, E IV, was cultivated in the normal way for 7 days in broth with rising antibiotic concentrations, upon which the usual cross testing was effected. The purpose was to find out the effect of rising concentrations and compare it with the foregoing, in which the concentrations remained constant throughout.

A study of the results (Table 42) shows that 1 $\mu\text{g/ml}$ of the antibiotic in broth kept in the refrigerator did not induce changes in the sensitivities to the primary antibiotics, nor any simultaneous changes to the other antibiotics.

The 24-hour cultivation (E II), on the other hand, resulted in changes both towards the primary antibiotics and simultaneous

TABLE 42

EFFECT OF CULTIVATION PERIODS OF DIFFERENT LENGTHS AND OF DIFFERENT CONCENTRATIONS OF ANTIBIOTICS ON THE INCREASE IN RESISTANCE OF *E. COLI* STRAIN 4 TO THE PRIMARY ANTIBIOTIC AND ON THE ACCOMPANYING SIMULTANEOUS CHANGES IN SENSITIVITY TO THE OTHER ANTIBIOTICS.

E I, 24 hours in refrigerator, concentration of primary antibiotic in broth 1 $\mu\text{g/ml}$
 E II, 24 » incubator, — » —
 E III, 7-day cultivation, — » —
 E IV, 7-day cultivation in broth with rising concentrations of the antibiotic.

		Sensitivity ($\mu\text{g/ml}$)			
		Aureo- mycin	Chloro- mycetin	Terra- mycin	Dihydro- strept.
Initial sensit.		3.9	7.8	1.9	31.25
Cultiv. in broth containing					
E I	Aureo- mycin	3.9	7.8	1.9	31.25
E II		7.8	31.25	3.9	15.6
E III		31.25	62.5	7.8	7.8
E IV		125	125	15.6	3.9
E I	Chloro- mycetin	3.9	7.8	1.9	31.25
E II		7.8	15.6	3.9	31.25
E III		62.5	125	3.9	15.6
E IV		62.5	>500	31.25	1.9
E I	Terra- mycin	3.9	7.8	1.9	31.25
E II		3.9	7.8	1.9	15.6
E III		15.6	62.5	15.6	15.6
E IV		62.5	250	15.6	3.9
E I	Dihydro- strept.	3.9	7.8	1.9	31.25
E II		1.9	7.8	1.9	>500
E III		1.9	7.8	0.97	>500
E IV		0.48	7.8	0.48	>500

slight shifts in sensitivity to the other antibiotics. It is obvious that the bacteria must grow in the broth containing the antibiotic before these changes occur.

The 7-day cultivation (E III in broth with 1 $\mu\text{g/ml}$ antibiotic)

resulted in an increase in resistance to the primary antibiotic. Similar shifts in sensitivity to the other antibiotics were, in part, greater than with part E II. The differences between the increases in sensitivity were smaller.

Part E IV was cultivated in broth with rising antibiotic concentrations. Increases in resistance to the primary antibiotic were greatest in this group, as were the simultaneous changes in sensitivity to the other antibiotics.

A review of the increases in sensitivities to dihydrostreptomycin acquired by cultivation in broth containing aureomycin, chloromycetin and terramycin, as well as of the simultaneous increases in sensitivity to aureomycin, chloromycetin and terramycin resulting from cultivation in broth containing dihydrostreptomycin, revealed that the increases in sensitivities were slight with all parts of the strain, but in the author's opinion some small difference could be detected between the increases of each group. Hence, the increases in sensitivity were greater with part E III than with part E II, and greater with part E IV than with part E III.

Summary

The purpose was to ascertain whether the length of cultivation period and the antibiotic concentration were of importance when *E. coli* was cultivated in broth containing one antibiotic and the simultaneous changes in sensitivity to the other antibiotics were observed.

1. It was found that *E. coli* had to grow in broth containing the antibiotic before there was any shift in sensitivity to the primary antibiotic or any simultaneous change in sensitivity to the other antibiotics.
2. Cultivation for a longer period in broth containing the antibiotic resulted in greater changes in sensitivity than when the cultivation was of shorter duration.
3. Cultivation in rising concentrations of the antibiotic resulted in greater sensitivity changes than cultivation in broth in which a constant small amount of the antibiotic was present.

5. DO ANTIBIOTIC COMBINATIONS PREVENT THE INCREASE IN RESISTANCE?

As the mode of action of dihydrostreptomycin on certain bacterial strains seemed to differ somewhat from that of aureomycin, chloromycetin and terramycin, the investigations were continued with the purpose of ascertaining whether this difference possibly manifests itself in preventing an increase in resistance when antibiotics of differing modes of action are used in combinations. *E. coli* strain 4 was employed as the test organism. It was cultivated for seven days in broth with the following antibiotic combinations:

1 $\mu\text{g/ml}$ aureomycin	+	1 $\mu\text{g/ml}$ dihydrostreptomycin	
1 » chloromycetin	+	1 »	»
1 » terramycin	+	1 »	»

It was found, firstly, that when after the third day a loopful of bacteria was transferred from the dihydrostreptomycin + terramycin combination to fresh broth (containing 1 $\mu\text{g/ml}$ dihydrostreptomycin + 1 $\mu\text{g/ml}$ terramycin), no turbidity could be seen in the tube the next day. Sensitivity determination showed the sensitivity to terramycin of this strain to have increased from 1.9 $\mu\text{g/ml}$ $\rightarrow$ 0.97 $\mu\text{g/ml}$. The same phenomenon was found to occur regularly in four control tests after 3—6 transfers. On the other hand, the resistance to terramycin of the same strain increased when the strain was cultivated by daily transfers in broth containing 1 $\mu\text{g/ml}$ terramycin alone (Table 43). In the same combination series, dihydrostreptomycin + terramycin, resistance to dihydrostreptomycin increased only slightly, although the dihydrostreptomycin resistance of the same strain increased 31.25 $\mu\text{g/ml}$ $\rightarrow$ 500 $\mu\text{g/ml}$ when 1 $\mu\text{g/ml}$ of dihydrostreptomycin without terramycin was administered daily. When no growth resulted with the combination dihydrostreptomycin + terramycin after 3—6 daily transfers, growth could be induced either by increasing the size of inoculum (1 loopful $\rightarrow$ 3 loopfuls) or by reducing the amount of terramycin from 1 $\mu\text{g/ml}$ $\rightarrow$ 0.5 $\mu\text{g/ml}$.

Sensitivity determinations effected after seven transfers from the combinations aureomycin + dihydrostreptomycin and chloromycetin + dihydrostreptomycin revealed that the resistance to dihydrostreptomycin had not increased to any considerable extent (31.25 $\mu\text{g/ml}$ $\rightarrow$ 62.5 $\mu\text{g/ml}$) in these combinations either. In the

TABLE 43

EFFECT OF ANTIBIOTIC COMBINATIONS ON THE INCREASE IN RESISTANCE OF
E. COLI STRAIN 4 TO DIFFERENT ANTIBIOTICS.

	Sensitivity ($\mu\text{g/ml}$)			
	Aureo- mycin	Chloro- mycetin	Terra- mycin	Dihydro- strept.
Initial sensitivity	3.9	7.8	1.9	31.25
After seven transf. in broth containing 1 $\mu\text{g/ml}$				
Aureomycin	31.25			
Chloromycetin		125		
Terramycin			15.6	
Dihydrostreptomycin ...				>500
Dihydrostreptomycin + aureomycin	15.6			62.5
Dihydrostreptomycin + chloromycetin		62.5		62.5
Dihydrostreptomycin + terramycin			0.97	62.5

combination 1 $\mu\text{g/ml}$ aureomycin + 1 $\mu\text{g/ml}$ dihydrostreptomycin, resistance to aureomycin grew 4-fold (in the same conditions, without dihydrostreptomycin, 8-fold). In the combination 1 $\mu\text{g/ml}$ chloromycetin + 1 $\mu\text{g/ml}$ dihydrostreptomycin an 8-fold resistance to chloromycetin was obtained (7.8 $\mu\text{g/ml}$ $\rightarrow$ 62.5 $\mu\text{g/ml}$), while in the same conditions without dihydrostreptomycin the resistance was 16-fold.

Summary

1. With the combination 1 $\mu\text{g/ml}$ aureomycin + 1 $\mu\text{g/ml}$ dihydrostreptomycin increased resistance of an *E. coli* strain to dihydrostreptomycin could be almost completely prevented, and the increase in resistance to aureomycin reduced.

2. With the combination 1 $\mu\text{g/ml}$ chloromycetin + 1 $\mu\text{g/ml}$ dihydrostreptomycin increased resistance of the same *E. coli* strain to dihydrostreptomycin, similarly, was almost completely prevented, and the increase in resistance to chloromycetin reduced.
3. With the combination 1 $\mu\text{g/ml}$ terramycin + 1 $\mu\text{g/ml}$ dihydrostreptomycin, similarly, increased resistance of the same *E. coli* strain to dihydrostreptomycin was almost completely prevented, and a slight increase was found in sensitivity to terramycin.

6. DISCUSSION

In the latter part of the investigation certain (comparative) experiments were carried out, closely related to the experiments in the former part.

From an *A. aerogenes* strain a strain was separated of which the sensitivity to dihydrostreptomycin exceeded that of the parent strain. Cultivation in broth containing aureomycin, chloromycetin and terramycin induced, with both strains, an increase in sensitivity to dihydrostreptomycin; the difference found between the sensitivities to dihydrostreptomycin of the two strains was approximately of the same extent as that between the initial sensitivities.

The duration of the increased resistance and increased sensitivity, detected by cross testing, was investigated by cultivating *A. aerogenes* strains of changed sensitivity in broth, effecting daily transfers for four weeks. It was not found that the increased resistance or increased sensitivity were reduced. As regards sensitivity, this denoted that the property in question in the *A. aerog.* strain employed could not be considered as temporary, as resulting from poor growth conditions spoiled by the antibiotic. On the other hand, the increased sensitivity decreased rapidly when the corresponding antibiotic was added to the broth.

All the experiments of the initial part of the investigation (Tables 7—31) were effected by using broth as the nutrient medium. Similar experiments, using different nutrient media, were carried out with one *E. coli* strain. The results were on the same lines and the changes in sensitivity mainly of the same range as in the experiments of the initial part. Hence, the nutrient media used seemed to have no significance in this respect, although the changes to primary antibiotics were slightly less in Kirchner medium.

Cultivation of *E. coli* for 24 hours in broth containing one antibiotic induced, not only increased resistance to this primary antibiotic, but also simultaneous changes in sensitivity to the other antibiotics. However, the changes were smaller than when *E. coli* was cultivated for 7 days in broth containing the primary antibiotic. When *E. coli* was kept in a refrigerator for 24 hours in broth containing the antibiotic, and no growth ensued, there were no changes in sensitivity.

When one *E. coli* strain was cultivated in broth containing a constant small amount of antibiotic, the simultaneous changes in sensitivity to the other antibiotics were smaller than when the same *E. coli* strain was cultivated in broth with rising quantities of antibiotics.

On the basis of the investigations in the initial part, the preventive effect, furthermore, of the following combinations of antibiotics on the increase in resistance was tested:

Dihydrostreptomycin	+ aureomycin
»	+ chloromycetin
»	+ terramycin

One *E. coli* strain was cultivated in the same amount of broth containing 1 $\mu\text{g/ml}$ dihydrostreptomycin + 1 $\mu\text{g/ml}$ aureomycin (or chloromycetin or terramycin) for 7 days. Resistance to aureomycin and chloromycetin increased less than when cultivated without dihydrostreptomycin. *Sensitivity to terramycin showed a tendency to increase.* The resistance to dihydrostreptomycin was only doubled in these combination experiments. *The different effect mechanism on E. coli of dihydrostreptomycin as compared with the aureomycin-chloromycetin-terramycin group manifested itself, hence, in partial or total prevention of the increase in resistance.* In the author's opinion the results indicated that the combinations dihydrostreptomycin + aureomycin (or chloromycetin or terramycin) do perhaps play some part in preventing resistance in vivo also. These combinations, moreover, have already been used successfully both in animal tests and therapy. Good results in the treatment of *Brucella melitensis* infections in mice were attained by Heilman (28) with the combination dihydrostreptomycin + aureomycin and by Werner and Knight (56) (36) with the combination streptomycin + aureo-

mycin or chloromycetin or terramycin. Using the same combination (streptomycin + aureomycin or chloromycetin or terramycin), H e r r e l l and B a r b e r (29) attained good results in the treatment of human brucellosis. Similarly, in the treatment of post-irradiation infections in mice, the best results were attained with the combination streptomycin + terramycin by H a m m o n d and M i l l e r (26).

VI Summary

1. The author, having cultivated bacteria in broth containing one antibiotic, has studied the possible simultaneous changes occurring in the sensitivities of bacteria to the other antibiotics. 27 bacterial strains were employed in the investigations, from the following 9 bacterial species: *E. coli*, *A. aerog.*, *S. typhi*, *Proteus vulgaris*, *Ps. aeruginosa*, *Staph. aureus*, *Staph. albus*, *Strept. pyogenes* and *B. subtilis*. The following antibiotics were employed: aureomycin, chloromycetin, terramycin, dihydrostreptomycin and penicillin.
2. Aureomycin, chloromycetin and terramycin, in their mode of action, seemed to constitute a special group in some way differing from dihydrostreptomycin. With the resistance of the bacteria of the species *E. coli*, *A. aerog.*, *S. typhi*, *Proteus vulgaris* and *Ps. aeruginosa* to any one of the antibiotics of this group increasing, a simultaneous increase in resistance followed to the two other antibiotics of the group. There were also indications of the sensitivity to dihydrostreptomycin increasing at the same time. When increased resistance to dihydrostreptomycin was induced sensitivities to aureomycin, chloromycetin and terramycin showed signs of a simultaneous increase.
3. Changes mentioned under Item 2 occurred also with the other bacterial species employed, but the results were dispersed.
4. Cultivation in broth containing penicillin was not found to induce changes in sensitivity to aureomycin, chloromycetin, terramycin and dihydrostreptomycin. Nor was cultivation in broth containing aureomycin, chloromycetin, terramycin or dihydrostreptomycin found to result in changes in sensitivities to penicillin.

5. On the basis of the results mentioned under Item 2, further experiments were carried out with an *E. coli* strain in order to discover the extent to which combinations of antibiotics prevent the increase in resistance. It seemed possible to prevent the increase in resistance, at least in part, in vitro, by using the following combinations:

1. Dihydrostreptomycin + aureomycin
2. » + chloromycetin
3. » + terramycin.

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Abbreviations according to British Abstracts.

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